Molecular Analysis of Fungal Pathogenicity in Crown Rot Disease of Wheat Caused by *Fusarium graminearum*

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Declaration by author

This thesis **is composed of my original work, and contains** no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Statement of Contributions to Jointly Authored Works Contained in the Thesis

Stephens, A. E., Gardiner, D. M., White, R. G., Munn, A. L. and Manners, J. M. (2008) Phases of infection and gene expression of *Fusarium graminearum* during crown rot disease of wheat. *Molecular Plant-Microbe Interactions,* **21,** 1571-1581. – White R.G contributed to the development of microscopy techniques and interpretation of histology. Manners J.M, Gardiner D.M and Munn A.L contributed to conception design and reviewing. Stephens A.E was responsible for the remainder of the work.

Desmond, O. J., Manners, J. M., Stephens, A. E., Maclean, D. J., Schenk, P. M., Gardiner, D. M., *et al.* (2008b) The *Fusarium* mycotoxin deoxynivalenol elicits hydrogen peroxide production, programmed cell death and defence responses in wheat. *Molecular Plant Pathology,* **9,** 435-445. – Gardiner D.M the generation of *Fg Tri5* deletion lines. Schenk, P. M and Maclean, D.J were responsible for critical reading, Manners J.M contributed to conception design and reviewing, Stephens, A. E was responsible for the experimental work, interpretation of data and writing for the estimation of fungal biomass in wheat stems. Desmond, O. J was responsible for the remainder of the work.

Statement of Contributions by Others to the Thesis as a Whole

Andrew Beacham contributed to FCR scoring in Chapter 5.

Donald Gardiner contributed to FCR and FHB assays.

Donald Gardiner, John Manners and Alan Munn contributed to critical reading and reviewing.

Statement of Parts of the Thesis Submitted to Qualify for the Award of Another Degree

None

Published Works by the Author Incorporated into the Thesis

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Abstract

Several *Fusarium* species can cause *Fusarium* head blight (FHB) and *Fusarium* crown rot (FCR) diseases in wheat and these are of economic importance in wheat production systems globally. *Fusarium graminearum* represents a model pathogen species to study these diseases because it has a sequenced genome, commercially available gene expression arrays and an growing collection of mutants impaired in pathogenicity and virulence, at least for FHB. FCR occurs at the stem base of the wheat plant, causing major reductions in grain yield. FCR has been much less intensively researched than FHB and the infection process of *F. graminearum* during crown rot disease in wheat has not been studied previously at the molecular level. Fungal biomass estimations by real-time quantitative PCR analysis of DNA from inoculated plants identified three distinct phases of infection during FCR, an initial increase in fungal mass in phase 1 up to 2 days post inoculation (dpi), then a reduction during phase 2 until 14 dpi followed by a large increase thereon in phase 3 that corresponded to symptom development. Histological characterisation of *F. graminearum* colonisation during these three phases of infection showed that initially the spores germinated on the stem surface at the point of inoculation forming a superficial hyphal mat. This occurred within the first two days of infection. The second phase was characterised by a period of low amounts of fungal tissue present in the infected plants and 14 days following infection hyphae were only observed below the point of inoculation at the stem base of the wheat seedling and had penetrated and colonised the adaxial epidermis of the outer leaf sheath. Following this, the third phase was characterised by a major colonisation of the internal tissues of the crown which corresponded to visible symptom development around 35 days after inoculation.

Fungal gene expression during all three phases of infection were examined using the Affymetrix GeneChip system comprised of 22,000 *F. graminearum* gene probe sets. This analysis showed 1,839 genes were significantly up regulated *in planta* compared to axenic vegetative mycelia, including some known FHB virulence genes (e.g. those involved in the biosynthesis of trichothecene toxins). Fungal genes differentially regulated between the phases were identified indicating that FCR disease development requires a coordinated process involving distinct fungal gene expression programs. A bioinformatic comparison of global *F. graminearum* gene expression during FCR of wheat with published data for FHB of barley indicated similarities at very early stages of infection but divergence thereafter.

It was decided to functionally test whether *F. graminearum* utilises the same virulence genes in FCR and FHB diseases. Because no virulence genes have been previously identified from FCR

studies a small group of genes were initially selected from the FCR gene expression studies for further functional analysis using gene knock-out technology. Only two of these genes showed a changed phenotype during *Fusarium* infection of wheat plants and they encoded a probable ABC transporter (*FgABC1)* and a probable superoxide dismutase (*FgSOD1)*. It was interesting to note that even though both *FgABC1* and *FgSOD1* exhibited similar transcription profiles during both FCR of wheat and FHB of barley it was found that *FgABC1* was specifically required for full FCR disease development on the wheat cultivar Kennedy whereas *FgSOD1* was specifically required for FHB disease on the same cultivar. This indicated that *F. graminearum* virulence genes can show specificity to the infection of different plant tissues and that these types of genes cannot be predicted based only on their transcription profiles. It is suggested that *F. graminearum* induces a global set of virulence factors but only some of these may be effective in particular tissues.

To test further whether there was tissue specialisation for specific tissues and FCR & FHB diseases, a group of *F. graminearum* genes that were known virulence factors during FHB were tested to see if they were also virulence factors for FCR. This analysis showed that two genes displayed specificity only for FHB and five were virulence factors for both FHB and FCR. One of the genes that was a virulence factor for both diseases was the *Tri5* gene that is necessary for the biosynthesis of trichothecene mycotoxins. This gene and these toxins did not appear to be necessary for symptom development and the induction of host defence responses but were necessary for fungal colonisation of the crown and stem in later stages of infection. Interestingly there were parallels in the role played by the *Tri5* gene in FCR and that reported for FHB where it is necessary for colonisation for the spike.

This study is the first molecular analysis of any *Fusarium* species during crown rot of wheat. Importantly, it shows that there may be specialisation towards host tissues for some virulence genes but also suggests that some factors may be non-specifically required for infection and it is these factors that will represent attractive targets for future control measures of both diseases.

Keywords

Fusarium graminearum, head blight, crown rot, Deoxynivalenol, pathogen, Superoxide, ABC transporter, mycotoxin

Australian and New Zealand Standard Research Classifications (ANZSRC)

070603 Horticultural Crop Protection (Pests, Diseases and Weeds) 50%, 060505 Mycology 30%, 060405 Gene Expression (incl. Microarray and other genome-wide approaches) 20%

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List of Abbreviations

FCR	*Fusarium* crown rot
FHB	*Fusarium* head blight
ROS	Reactive oxygen species
H_2O_2	Hydrogen peroxide
SOD	Superoxide dismutase
Cu-Zn	Copper Zinc
O_2^-	Superoxide
DON	Deoxynivalenol
ABC	ATP-binding cassette
EST	Expressed sequence tag
RT-qPCR	Real-time reverse-transcriptase polymerase chain reaction
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
mRNA	messenger RNA
dpi	Days post inoculation
bp	Base pairs
kb	Kilobase pairs
min	minute
h	hour
Wt	Wild type
SNA	Synthetic nutrient agar
PDA	Potato dextrose agar
GM	Genetically modified
Cv	Cultivar

CHAPTER 1: Introduction and Literature review

Global importance of wheat

Wheat (*Triticum aestivum*) is a domesticated grass that has been cultivated around the world for thousands of years. The grain produced by the wheat plant serves as a staple food for people in many countries due to its high content of protein, carbohydrate, dietary fibre, fat and iron. Over the five-year period of 2003-2007, an average of over 605 million tonnes of wheat was produced annually around the world by 124 different countries. Wheat was the third largest cereal produced after maize and paddy rice see Table 1.1.

Table 1.1: Global Cereal Production 5-year average (2003-07) (Food and Agriculture Organization of the United Nations http://faostat.fao.org).

Cereal	Production (million tonnes)
Maize	714
Rice, paddy	624
Wheat	**605**
Barley	142
Sorghum	60
Millet	32
Oats	25
Rye	15

China, India and the United States of America were the top three wheat producers while Australia was the 10th largest with a mean production of over 19 million tonnes (Table 1.2A). Wheat is the biggest cereal crop produced in Australia (Table 1.2B), but the period of 2003-2007 contains results from several poor years because of the drought in 2006 and 2007. Australian wheat production during more favourable conditions in 2003-2005 ranged from 21-26 million tonnes per annum. Importantly, Australia is considered one of the top three wheat exporters in the world, alongside the USA and Canada (Figure 1.1). Therefore biological and environmental factors that decrease wheat production in Australia can have significant flow-on effects on wheat availability and pricing in the world market. It is clear that the drought years of 2006-07 had a major effect on wheat exports in Australia where they dropped by half (Figure 1.1).

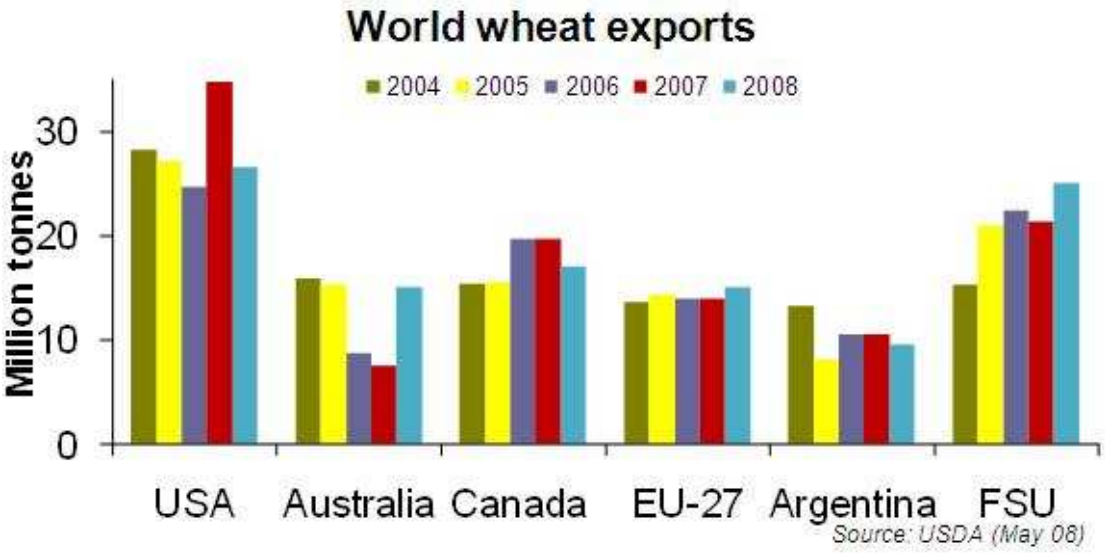

Figure 1.1: Top 5 wheat exporters for the last 5 years. (www.hgca.com)

Table 1.2: (A) Global Wheat Production 5-year average (2003-07) and (B) Australian cereal production 5-year average (2003 – 07) (http://faostat.fao.org).

A.

Country	**Production** (million tonnes)
1. China	98
2. India	70
3. USA	56
4. Russian Fed	44
5. France	35
6. Canada	24
7. Germany	22
8. Pakistan	21
9. Turkey	20
10. Australia	**19**
11. United Kingdom	14
12. Iran	14
12. Argentina	14
13. Ukraine	13
14. Kazakhstan	12
15. Poland	8
16. Egypt	7
17. Italy	7
18. Spain	6
19. Uzbekistan	6

B.

Cereal	**Production** (million tonnes)
Wheat	**19**
Barley	7.6
Sorghum	1.7
Oats	1.3
Triticale	0.5
Rice, paddy	0.5
Maize	0.3
Millet	0.03
Rye	0.02

In Australia, wheat is grown largely within a zone termed the 'Australian wheat belt'. The wheat belt runs south from southern QLD extends west to SA and the southern portion of WA (Figure 1.2). Different varieties of wheat are grown in different parts of the wheat belt depending on summer or winter dominant rainfall patterns and other climatic and environmental factors.

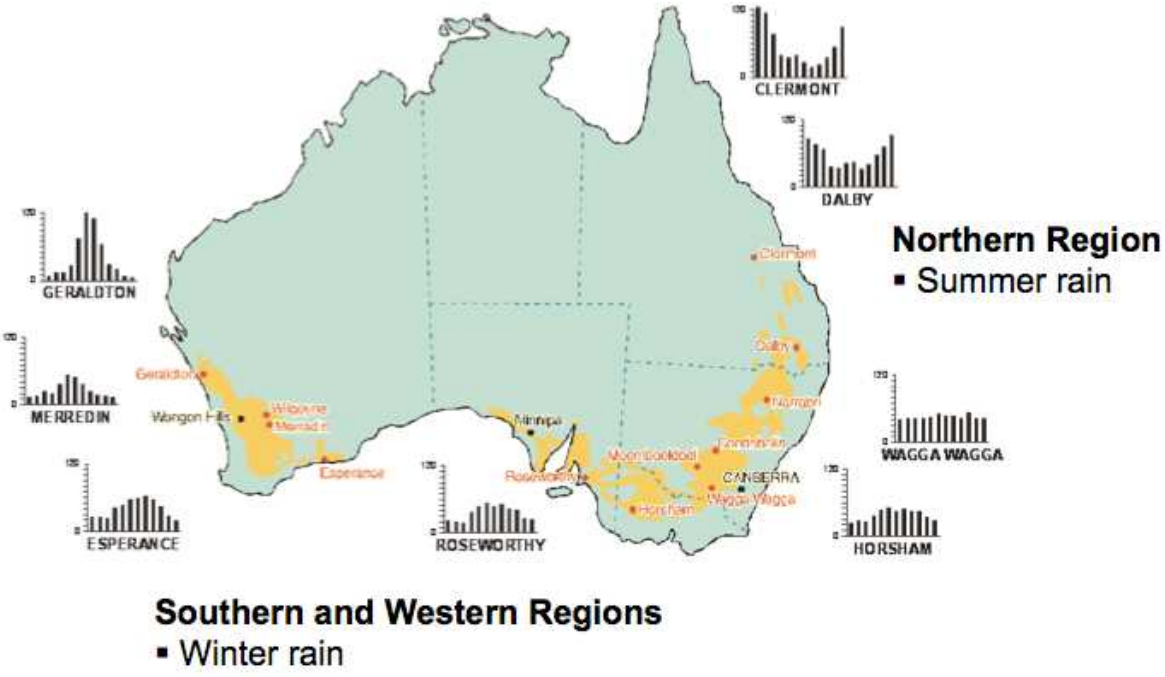

Figure 1.2: Map of Australia showing the Australian wheat belt in yellow. Small graphs show average monthly rainfalls from January to December that indicate summer dominant rain in the northern parts and winter dominate rain in the southern and western regions.

Wheat is an important agricultural product around the world but as with most crops it is susceptible to many different diseases that reduce grain yield and quality. In Australia it is estimated that wheat diseases currently cause an annual loss of $914M (Table 1.3) and if current measures were not in place to manage these diseases the potential losses are estimated to be $3.15B for the top 5 disease alone (Murray & Brennan, 2009). Common fungal-incited wheat diseases include; rusts (*Puccinia spp.* and *Thalictrum* spp), Septoria diseases (*Stagonospora nodorum* and *Mycosphaerella graminicola*) and Yellow spot (*Pyrenophora tritici-repentis* (Ptr)). But some of most devastating wheat diseases are caused by various species of *Fusarium*.

Table 1.3: Current losses estimated for wheat diseases in Australia. * other diseases include stem rust, leaf rust, take-all, bare batch, common root rot, wheat streak mosaic virus and barley yellow dwarf virus (Murray & Brennan, 2009).

Disease	$/ha	$ Million
Yellow spot	17.82	212
Stripe rust	10.62	127
Septoria nodorum blotch	9.07	108
Crown rot	6.63	79
Pratylenchus neglectus	6.13	73
Losses from other diseases*	16.37	314
TOTAL	$76.64/ha	$914M

Fusarium incited diseases of wheat

In Australia *Fusarium* spp. can cause two different types of disease in wheat; they are termed *Fusarium* head blight (FHB) and *Fusarium* crown rot (FCR). FHB involves the infection of the wheat flower at anthesis while FCR involves infection of the stem base and crown of the wheat plant at any stage in plant growth after germination. In Australia, three *Fusarium* spp. are the main causal agents for *Fusarium* diseases in wheat; *F. graminearum*, *F. pseudograminearum* and *F. culmorum*. FHB and FCR can be caused by the same *Fusarium* species (Marasas *et al.*, 1988), but particular species of the fungus have a propensity to infect either the head or the crown of wheat plants (Akinsanmi *et al.*, 2004). In a survey of wheat in eastern Australia *F. pseudograminearum* was most frequently associated with FCR disease and *F. graminearum* more frequently associated with FHB but both pathogens were also associated, albeit at lower frequencies, with the alternate disease (Akinsanmi *et al.*, 2004). In other countries FHB has been reported as being the most economically important disease, but in Australia FCR is more important.

This literature review focuses on the fungal-plant interaction of *Fusarium*-incited diseases in wheat. It will initially compare general aspects of the pathology of the two diseases FHB and FCR and then discuss aspects of the molecular biology of *F. graminearum* pathogenicity.

A. B.

Figure 1.3: Example of (A) FCR disease symptoms at the stem base and (B) FHB symptoms of wheat (left) with a non-infected wheat head (right)

COMPARATIVE PATHOLOGY OF FHB AND FCR

Initially the general pathology of FHB and FCR will be reviewed in terms of factors that influence symptom and disease development.

FUSARIUM HEAD BLIGHT

Fusarium head blight, is usually referred to as *Fusarium* head scab in North America, and its symptoms include bleached/dried-out looking florets or glumes to black lesions, shrivelled up grains and buckled awns that usually bend downward (Figure 1.3B). During FHB, the wheat grains become desiccated due to the degradation of starch granules and therefore grain weight and seed fertility are reduced (Jackowiak *et al.*, 2005). Wet and warm weather conditions at anthesis favour FHB infections (McMullen *et al.*, 1997) (Bai & Shaner, 1994). *F. graminearum* is the most

common *Fusarium* species to incite FHB infection in wheat in Australia (Akinsanmi *et al.*, 2004), North America and many other parts of the world (Goswami & Kistler, 2004, O'Donnell *et al.*, 2000). Like many other fungi in the phylum Ascomycota, *F. graminearum* can reproduce both sexually and asexually. Therefore, across the published literature this fungus is referred to as both the asexual anamorph *F. graminearum* and the sexual teleomorph *Gibberella zeae* (Trail *et al.*, 2005, Goswami & Kistler, 2004). Two different spores can be produced by *F. graminearum*, macroconidia are formed by asexual reproduction and have a distinctive fusiform or banana shape. Ascospores are formed from sexual reproduction and are produced within a perithecia fruiting body, ascospores are considerably smaller than macroconidia and have a different shape. Because the two types of spores have distinct morphology and dispersal mechanisms, they aid different types of infection and disease epidemiology.

The primary inoculum of FHB in the USA is from ascospores, whereas in Australia it is from macroconidia (Mitter *et al.*, 2006a). Ascospores have larger dispersion areas than macroconidia because they are forcibly discharged from the perithecia and they are able to travel on the wind, while macroconidia rely more on physical contact and splash dispersal (Fernando *et al.*, 1997). The dominance of macroconidia as inoculum in Australia may explain the localised nature of FHB epidemics (Akinsanmi *et al.*, 2004). FHB infection begins with the contact and adhesion of spores to the wheat head during flowering, then the spores germinate and the germ-tubes grow along the plant surface towards the inside of the flowering spikelets where it penetrates into soft tissue (Kang *et al.*, 2005). After penetration, the fungus spreads from spikelet to spikelet usually via the vascular bundles within the rachis (Ribichich *et al.*, 2000). As a consequence the xylem and phloem can become damaged with occlusions forming that restrict the flow of nutrients to the top of the head and this causes tissue bleaching and death (Ribichich *et al.*, 2000). In FHB, initial bleaching can be seen within days after infection and full symptoms are exhibited within 1-2 weeks. The colonisation pathway of *F. graminearum* during FHB has been best characterized using GFP-expressing strains of *F. graminearum* where fungal colonisation of the rachis has been shown as the mechanism by which spread occurs through the spikelet (Jansen *et al.*, 2005, Gardiner *et al.*, 2009b). GFP-expressing strains have also been used to delineate the role of mycotoxins in the infection pathway and this is described later.

FUSARIUM CROWN ROT

Fusarium crown rot is occasionally referred to as foot-rot and its symptoms include necrotic dark lesions of the crown and basal stem tissue (Figure 1.3A). In severe cases of FCR a common symptom is the development of 'white heads', which are spikes that prematurely dry out and have no grain. Although symptoms mainly appear at the base of the plant, very severe FCR epidemics can completely eliminate grain yields in Australia (Southwell *et al.*, 2003) and yield reductions can be as severe as 35-61% in America (Smiley *et al.*, 2005). Although small amounts of grain may be damaged due to rare occurrences of fungal colonisation (Mudge *et al.*, 2006), it is more likely that the infection inhibits the flow of water and nutrients from the roots to the head. Dry and hot weather conditions appear to favour FCR infection and symptom development (Cook, 1981). In Australia *F. pseudograminearum* is the principal FCR pathogen, but it has been shown that *F. graminearum* and *F. culmorum* can infect both the crown and wheat base (Aoki & O'Donnell, 1999, Backhouse *et al.*, 2004).

The primary inoculum for FCR are the macroconidia that develop on the infested stubble from the preceding year and this inoculum can remain viable for up to two years in the ground litter (Burgess, 2005). As the wheat seedlings emerge from the soil it is hypothesised that the stem base may come in physical contact with the ground litter facilitating the transfer of spores to the plant or rain may deliver spores from ground litter to wheat seedlings via splash dispersal (Figure 1.4). In the field it has been shown that crown rot can occur in a wheat plant at any growth stage (Summerell *et al.*, 1989, Burgess *et al.*, 1981, Purss, 1966) and it has been suggested that when stubble has been cultivated into the soil then infection occurs lower in the stem base compared to when the stubble is left above ground (Summerell *et al.*, 1990).

From the evidence gathered it seems that the high throughput glasshouse crown rot assay method outlined by Mitter *et al* (2006b) serves as a good model for investigating the host-pathogen interaction and mimicking field infection conditions. Briefly, this method uses macroconidia suspended in a drop of water to inoculate the stem base of two-week-old wheat seedlings. This method is non-invasive and replicates the natural inoculation of stems by splash dispersal. Because of the current no-till methods in modern cropping, it is reasonable to use an inoculation system that facilitates fungal penetration at the stem base above ground level. The method of Mitter *et al.*, (2006b) is thought to simulate field infection and plant response because the ranking for disease sensitivity of wheat cultivars provided by the bioassay matches that derived from field evaluations (Mitter *et al.*, 2006b).

The current understanding of the infection process during FCR induced in the glasshouse bioassay is as follows (Desmond, 2008, Desmond *et al.*, 2008a); fungal spores germinate on the surface of the stem base around 3-6 hours post-inoculation (hpi) and a superficial mycelial mat then develops and the fungus most probably penetrates the plant epidermis through the stomata within 24 hpi. Thus far, there has been no histological analysis of the interaction with the host after the fungus enters the basal stem tissue. Because the plant does not exhibit any visible symptoms or necrosis within the first 14 days post-inoculation, it is possible that the fungus at this point is biotrophic, and has not yet reached the necrotrophic phase. When visible symptoms such as necrosis become apparent after a few weeks to months post-inoculation, the fungus can be seen to have colonised the stem pith with minimal invasion of the vascular system (Mudge *et al.*, 2006). It is known that the fungus continues to colonise the wheat stem vertically over time although hyphal density is much reduced in upper nodes and organs.

There have been some studies of host defence responses during the development of FCR disease of wheat caused by *F. pseudograminearum* (Desmond, 2008, Desmond *et al.*, 2005). Once the wheat plant has recognized the invading *Fusarium* pathogen during FCR, short and long-term host defence responses are triggered (Desmond, 2008). Microarray analysis has shown that there are extensive changes in wheat gene expression associated with this response with 1248 and 1497 host genes induced and repressed, respectively (Desmond *et al*., 2008a). Examples of some of the changes observed include a peroxidase gene (TaPERO) that has been linked to cell wall modification and reactive oxygen species metabolism in plants (Thordal-Christensen *et al.*, 1992). Also, a germin-like gene that encodes an enzyme with superoxide dismutase activity (TaGLP2a germin-like) is induced. This gene has also been implicated in defence against other fungal pathogens (Schweizer *et al.*, 1999). Other germin-like genes function in host resistance in barley (Zimmermann *et al.*, 2006). A suite of other wheat PR genes are also up-regulated during FCR, homologues of which in other plants have been shown to be involved in plant defence (Desmond *et al*., 2005, Muthukrishnan *et al.*, 2001). If wheat defences are induced by treatment with methyl jasmonate prior to inoculation then reduced lesion development is observed indicating that induced defences may slow down disease progression at the early stages of infection (Desmond *et al*., 2005).

Toxin production by *Fusarium* spp.

Several toxins are produced by *F. graminearum* including fusarin C (Wiebe & Bjeldanes, 1981), zearalenone (ZEA) (Caldwell *et al.*, 1970) and the trichothecene toxin deoxynivalenol (DON) (Vesonder *et al.*, 1973). Zearalenone was previously known as F-2 toxin and is an oestrogenic mycotoxin synthesized through a polyketide biosynthetic pathway and has been linked to reproductive disorders in livestock (pigs and cattle) that ingest contaminated feed (Mirocha *et al.*, 1969) (Mirocha *et al.*, 1968). Zearalenone is mainly produced post-harvest in fungal contaminated grain stored in the presence of moisture and fluctuating temperature (Kuipergoodman *et al.*, 1987). In contrast, the trichothecene mycotoxins, e.g. DON, are produced during the infection process during both FHB and FCR diseases (Mudge *et al*., 2006).

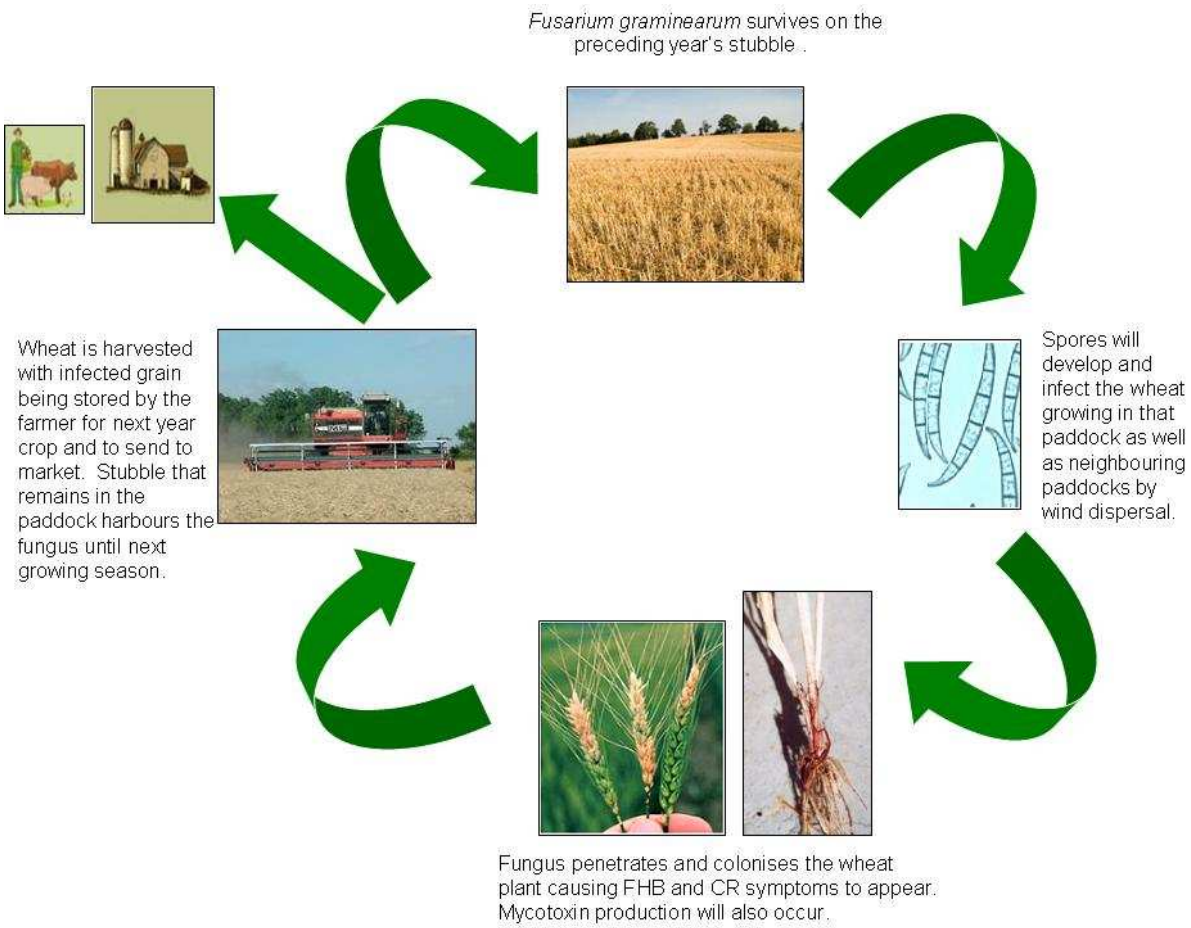

Figure 1.4: Flow diagram outlining the infection cycle of *Fusarium graminearum* on wheat.

The trichothecene mycotoxin DON is a powerful eukaryotic protein biosynthesis inhibitor (Wei & McLaughl, 1974, Ueno, 1984), therefore any DON contamination in wheat grain can be very dangerous to livestock and humans in downstream usage. Ingestion of DON can cause nausea, vomiting, seizures and death and it is also referred to as vomitoxin. In Europe and the USA, regulations have now been put in place to protect consumers against food products contaminated with the DON toxin. In Europe, the maximum quantity of DON in unprocessed cereals other than durum wheat, oats and maize is 1250 ppb as specified by the European Commission Regulation (EC) No 856/2005 (2005). Currently, Australia does not have any regulation regarding DON content in cereals, but must comply with international standards for its wheat exports.

Large gene clusters seem to be a common arrangement for genes involved in the biosynthesis of fungal derived plant pathogenic toxins as well as many other secondary metabolites and this also holds true for the genes involved in the biosynthesis of DON by *F. graminearum* (Yu & Keller, 2005). Through knock-out mutations, three DON-biosynthetic genes have been shown to be involved in the virulence of *F. graminearum* on wheat. These include *Tri5*, which encodes the enzyme responsible for the first step in the toxin biosynthetic pathway (Proctor *et al.*, 1995), *TRI6* which encodes a transcriptional regulator (McDonald *et al.*, 2005) and *TRI14* whose role is unknown but is required for *in planta* toxin production (Dyer *et al.*, 2005). The biosynthetic pathway is shown in Figure 1.5.

The colonisation pathway of *F. graminearum* during FHB has been best characterized using GFP-expressing wild type and *Tri5* mutants that are unable to produce trichothecene mycotoxins such as deoxynivalenol (DON) (Jansen *et al.*, 2005). During a FHB assay the non-DON producing *F. graminearum* mutant was not able to penetrate past the rachis node at the base of the floret to enter the rachis and spread throughout the spikelet, thus DON is conceivably acting as a colonisation factor during FHB enabling passage through the rachis. It was postulated that the non-DON producing strain of *F. graminearum* was unable to penetrate past the rachis node due to a thickening of the cell walls and the formation of a physical barrier. When the spike was infected with the wild type no cell wall thickening was observed, therefore suggesting that the DON toxin directly or indirectly inhibits the formation of this physical barrier.

As discussed previously, the DON toxin has been shown to be involved in facilitating the colonisation during both FHB and FCR infections. Importantly, it has been discovered that specific amines within the polyamine biosynthesis pathway can trigger the production of DON when

independently introduced to an *F. graminearum* axenic culture (Gardiner *et al*., 2009b). In addition, the same study also noted that DON production was repressed or induced in mixtures of different compounds indicating that DON-synthesis can be regulated by complex and varying environmental conditions. Considering that FHB and FCR occur in radically different tissue types it can be seen as an advantage that there are a number of cues that can induce the production of the DON toxin.

DON appears to induce programmed cell death in wheat plants, which may help to explain how it facilitates infection (Desmond *et al.*, 2008b). Studies of wheat gene expression following fungal challenge have suggested that reactive oxygen species (ROS) are generated at early stages in the interaction (Desmond *et al*., 2008a). It is interesting that in response to the presence of the fungal derived toxin DON, the wheat plant produces hydrogen peroxide (H_2O_2) as part of a ROS response and H_2O_2 production has been suggested as a signal for programmed cell death in plants (Desmond *et al*., 2008b).

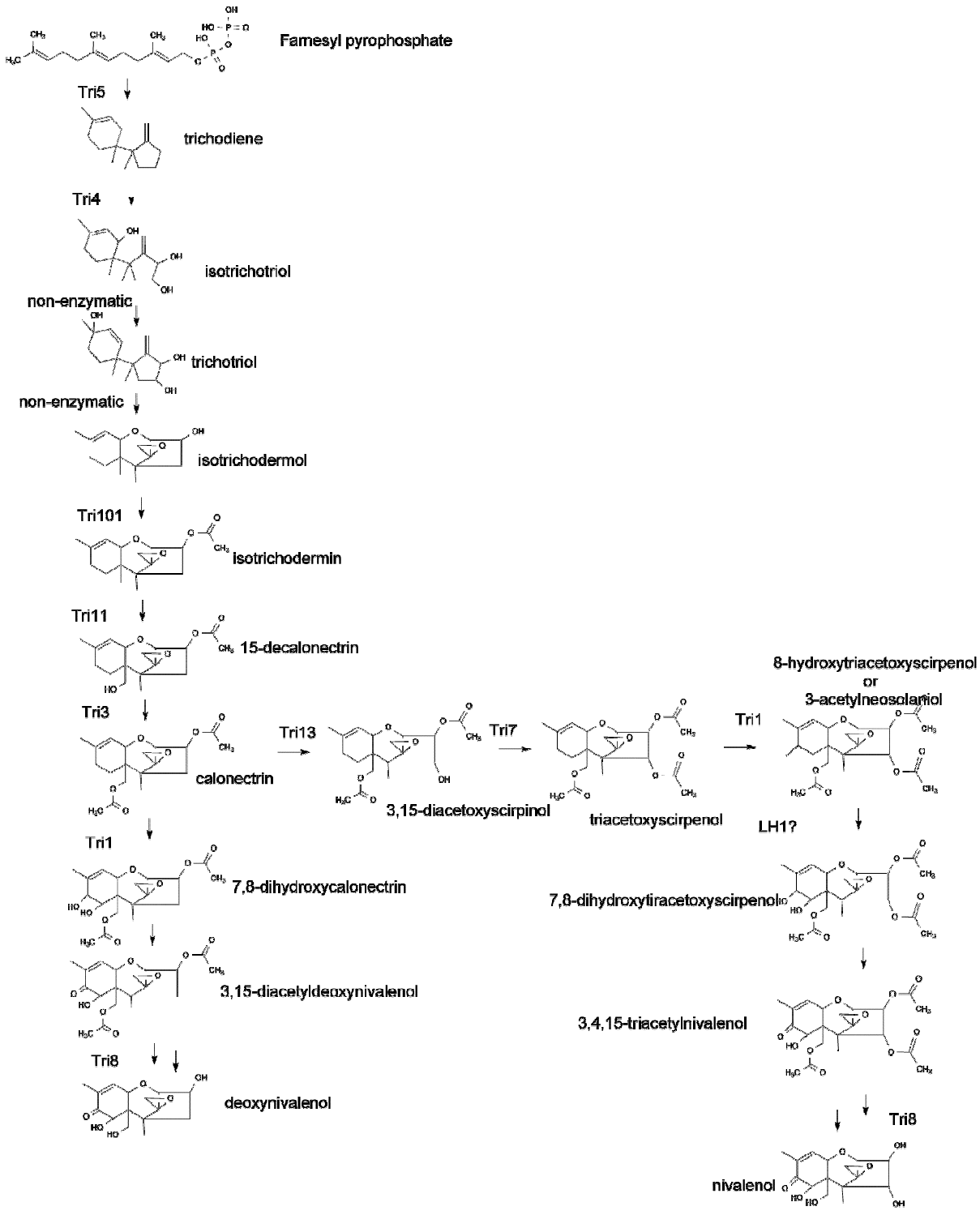

Figure 1.5: Representation of the *F. graminearum* trichothecene toxin biosynthesis pathway, including DON and nivalenol. Figure modified from (Desjardins, 2006).

Current management strategies for *Fusarium* disease in wheat

PRACTICES

To manage FHB and FCR in the field, grain growers employ several strategies that are briefly discussed here. Because the inoculum for both FHB and FCR comes predominantly from the ground litter and can remain viable for several years, the ground litter can be treated in a number of ways. Burning the residual stubble of a harvested crop can reduce the *Fusarium* spores/inoculum remaining in the paddock, this leads to less disease in the next years crop. Cultivating the stubble also decreases the amount of inoculum albeit to a lesser extent (www.grdc.com.au). Unfortunately, cropping practices such as burning-off and tilling do not meld well with contemporary farming practices. Burning off lowers the air-quality, contributes carbon dioxide to the atmosphere and reduces the water content within the soil. Nowadays low-till practices are favoured for moisture retention and preservation of soil structure.

Crop rotation can decrease the amount of viable inoculum present. This is where different crops that are not susceptible to *Fusarium* disease are rotated between the years when wheat is grown (Fernandez *et al.*, 2007). Such *Fusarium* resistant crops include pea and canola, which have been shown to decrease the amount of inoculum in stubble (Golkari *et al.*, 2008). There is an area of research that focuses on developing biocontrol agents for *Fusarium* disease that suggests that other microbial organisms introduced to stubble residue may compete with the saprophytic *Fusarium* and reduce its vegetative growth and spore formation. The addition of *Microsphaeropsis* sp. to wheat residue have been reported to reduce the formation of perithecia and ascospores (Bujold *et al.*, 2001) and it has been suggested that some *Trichoderma* spp. can inhibit saprophytic growth on stubble (Fernandez, 1992) although results can be variable. None of these experiments included conidia of *Fusarium* pathogens, which is an area of study that could be pursued in regards to the incidence of *Fusarium* crown rot in Australia. Other bacterial strains such as *Lysobacter enzymogenes* have been tested as biological control agents applied directly to the flowering head of the mature wheat plant with some success (Jochum *et al.*, 2006), but more research is needed to prove the efficacy of this FHB control technique especially if the grain is destined for human consumption. Before planting, seed can be fungicide-treated as a method of *Fusarium* disease control but this can also have an unwanted effect on germination and height of the plants (Garcia Junior *et al.*, 2008). Fungicides applied to the wheat head during flowering to control FHB have

proven to be expensive and not totally or consistently effective (Jones, 2000). In Australia 20% of the control measures taken for *F graminearum* come from breeding and 80% come from cultural practices (Murray & Brennan, 2009). Generally, in Australia where wheat is grown at low density across large areas of land the application of fungicides or biocontrol agents is not usually economically feasible unless applied in seed dressings at planting.

Other cropping factors can also affect the incidence of *Fusarium* disease and DON accumulation. For example, the incidence of FHB can be reduced with a lower seeding rate (less seeds planted per m^2) or low tillering plants because of reduced humidity in the canopy and an increased flowering period (Schaafsma & Tamburic-Ilincic, 2005) but this can also result in less grain harvested and higher evaporation of moisture from the soil. In general, some cropping techniques can greatly aid the production of a healthy disease free wheat crop, but these may not be practical in all situations. Because of the difficulties in using management to control *Fusarium* diseases in wheat there has been considerable interest in developing genetic resistance.

Host Resistance and Tolerance

Different varieties of wheat can vary quantitatively in their susceptibility to *Fusarium* spp. and therefore host resistance can provide another approach to managing the disease. Currently, there are no commercial varieties of wheat that are completely resistant to *Fusarium* diseases. For FHB, the Chinese derived wheat cultivar Sumai 3 provides resistance to spread of the disease from the initially infected floret (Bai & Shaner, 1994) (VanGinkel *et al.*, 1996), but provides little to no resistance to FCR (Liu *et al.*, 2004). Several QTLs (quantitative trait loci) for resistance to FHB have been identified through the analysis of mapping population and deletions lines, these include 3BS, 3A, 5A, and 6B (Ma *et al.*, 2006, Miedaner *et al.*, 2006, Yang *et al.*, 2003) and wheat breeders aim to introgress these into many cultivars destined to be grown in FHB-prone environments (Bai & Shaner, 2004). These and other FHB resistance QTL marker assisted selection analyses of wheat have been reviewed by Buerstmayr (2009). The 3BS QTL for resistance to FHB derived from Sumai3 coincides with a QTL for the detoxification of the trichothecene mycotoxin deoxynivalenol and the resistance appears to operate by preventing spread of the fungus which is consistent with what one might predict would happen by countering the action of trichothecenes during infection (Lemmens *et al.*, 2005). However the genes underlying the 3BS resistance locus have not been

cloned yet and the full gene complement that underpins this resistance locus for FHB is not completely understood.

In Australia, the cultivar Sunco (Bariana *et al.*, 2001) provides some tolerance to FCR but by no means is it considered fully resistant. Several QTLs have been identified for FCR tolerance including 1AL, 1DL, 2DL, 4BL and 5DS (Bovill *et al.*, 2006, Collard *et al.*, 2006, Wallwork *et al.*, 2004). It has been shown that defence genes in Sunco are expressed to a much higher degree than in another wheat cultivar that is more susceptible to *Fusarium* both before and after exposure to the fungal pathogen, and this may contribute to its partial *Fusarium* resistance (Desmond *et al*., 2005). There are ongoing efforts to identify QTLs that can be recombined to construct stronger resistance to FCR. Durum wheat is more highly susceptible to both FCR and FHB than bread wheat and there are few resistance sources available in a tetraploid background and most resistance breeding strategies are focused on introgression from hexaploid resistance sources (Daniel & Simpfendorfer, 2008).

Because traditional wheat breeding is a very time consuming process taking a decade or more to produce new varieties, the production of genetically modified (GM) cultivars of wheat that express fungal resistant traits is very appealing but also controversial. The multinational agricultural biotechnology companies, Monsanto and Syngenta have reported that they have GM wheat in the pipeline and are completing trials or waiting for market acceptance for its release. In 2004, Monsanto reportedly halted plans to release a GM cultivar of wheat that was resistant to herbicide due to foreign buyers being unwilling to use GM wheat for human consumption (http://news.bbc.co.uk/1/hi/business/3702739.stm). The Swiss-based company Syngenta also has a GM wheat cultivar that has been developed to have resistance against *Fusarium* diseases (http://www.foodnavigator.com/Financial-Industry/Syngenta-moves-closer-to-launching-GM-wheat). Apart from attempts by the big commercial agricultural biotechnology companies to produce a *Fusarium* resistant GM cultivar of wheat, smaller research laboratories have published their attempts many of which are reviewed in (Dahleen *et al.*, 2001) and summarized in Table 1.4. Genes that encode proteins that add to the hosts existing defence systems, degrade the fungal specific structural components such as cell walls and membranes, interfere with fungal metabolism or interfere with fungal pathogenicity programs can be introduced into the wheat genome. Conversely, deleting or silencing native genes that negatively regulate host defences active against necrotrophs like *F. graminearum* may increase resistance. These include genes that are involved in recognising the invading pathogen and inciting programmed cell death responses.

Table 1.4: Summary of published strategies for engineering resistance/tolerance to *Fusarium* and its toxins in wheat and other *Fusarium*-susceptible plants.

Published GM strategy	Reference
Expression of defence genes in wheat that increase resistance to FHB. Glucanase, purothionin and thaumatin-like protein.	(Mackintosh *et al.*, 2007)
Introduction of the *Arabidopsis thaliana* NPR1 gene in wheat has been shown to establish resistance to FHB infection.	(Makandar *et al.*, 2006)
Expression of wheat PR- proteins. Chitinase and glucanase.	(Anand *et al.*, 2003)
Insertion of a ZEA detoxification gene into maize	(Igawa *et al.*, 2007)
Insertion of *Fusarium* gene FsTRI101 acetyltransferase gene into wheat for detoxification of DON.	(Okubara *et al.*, 2002)

MYCOTOXIN MANAGEMENT STRATEGIES

Approaches to reduce the mycotoxin contamination that occurs in wheat products from *Fusarium* infection include a number of post-harvest treatments. Detoxification and biodegradation processes have been reported for the *Fusarium* mycotoxin ZEA that have been summarized by (Zinedine *et al.*, 2007). They include using H_2O_2 for the degradation of ZEA in contaminated corn (Abd Alla, 1997), extrusion cooking which is a heating process used to produce many breakfast cereals (Ryu *et al.*, 1999), the use of mixed cultures of bacteria to degrade ZEA by metabolizing the product (Megharaj *et al.*, 1997) and genetic modification of the susceptible host plant maize to enable detoxification of ZEA (Igawa *et al.*, 2007). Unlike ZEA, detoxification or degradation methods for DON decontamination have proven to be more difficult to optimize because the DON molecule is more heat stable and therefore it cannot be degraded through heat treatments of food products (Wolf-Hall *et al.*, 1999). There have been reports of soil borne microbial organisms degrading or bio-transforming DON such as a strain of *Aspergillus tubingensis* (He *et al.*, 2008), *Agrobacterium-Rhizobium* mixtures (Shima *et al.*, 1997) and also plant derived enzymes such as the UDP-glycosyltransferase found in *Arabidopsis thaliana* (Poppenberger *et al.*, 2003). However these latter approaches are also being applied in GM cereals to reduce disease development and toxin contamination.

Fusarium graminearum as a model pathogen

Fusarium graminearum is the model species studied in regards to *Fusarium* diseases in wheat, particularly at the molecular level. The *F. graminearum* genome has been sequenced and is ~36Mb in size comprising ~14,000 predicted genes with 4 chromosomes (Cuomo *et al.*, 2007). In 2003 the Broad Institute released the first of the *F. graminearum* (PH-1) genome sequences (NCBI accession AACM00000000), the current release of the genome is estimated to be at ~10X coverage. This resource lends itself to many applications that aid gene discovery and functional analysis, for example an Affymetrix GeneChip for *F. graminearum* has been validated and released (Guldener *et al.*, 2006b). All of the 14,000 predicted genes are represented on the chip, which allows global expression analysis of *F. graminearum* in a variety of environments, which facilitates investigation of the host-pathogen interaction at a molecular level (Guldener *et al*., 2006b). Most of the studies of *F. graminearum* have focused on its role in causing FHB disease but because it can also cause FCR disease (Mudge *et al*., 2006) it can also be used for the molecular analysis of FCR disease.

When the *F. graminearum* genome sequence was released, the Broad Institute used the Calhoun automated gene prediction scheme to produce an annotated genome (http://www.broad.mit.edu). A second annotation of the genome was developed by the Munich Information Center for Protein Sequences (MIPS, www.mips.helmholtz-muenchen.de/genre/proj/FGDB/) independently to the Broad annotation. This automated gene prediction used FGENESH which is a prediction system that uses a diverse range of fungal genomes as a reference such as *Ustilago maydis* and *S. pombe* (www.softberry.com). It is interesting to note that when the Affymetrix GeneChip was designed for this genome, multiple probe sets were used to identify the genes that had different annotation calls between the Broad and the MIPS versions and this has helped identify which annotation had the greater accuracy.

Studying the *F. graminearum* genome as a whole has allowed overall observations to be made about the structure of the genome and how this may influence its function. For example, it has been noted that the single nucleotide polymorphism (SNP) density increases in the telomeric regions of the genome. In addition to this, it was interesting to also note that there were several regions within three of the chromosomes that exhibited high SNP density indicating that these specific sites have possibly undergone sequence rearrangements (Cuomo *et al*., 2007). It is also noted that there are

less repetitive sequences and paralogous genes in the *F. graminearum* genome when compared to other filamentous fungi. It has been suggested that this may be due to a mechanism in *F. graminearum* and other ascomycetes called repeat induced point mutation (RIP), where point mutations are introduced into duplicated sequence for example on transposable elements as well as highly similar native genomic sequences during meiosis (Trail, 2009, Galagan & Selker, 2004, Cuomo *et al*., 2007).

At the outset of this project, several published *F. graminearum* Affymetrix GeneChip experiments have been posted on the publically available website database www.plexdb.org and the most notable experiments are summarized in Table 1.5. These experiments cover global expression by the fungus during a range of stages in the *F. graminearum* life cycle. Experiments FG5, FG6 and FG7 study global gene expression during spore development (sexual and asexual) and germination while FG5 and FG6 identified that the transcription of particular chloride, potassium and calcium ion transporter genes were specifically associated with the ascus discharge mechanism including the calcium ion channel *CCH1* (Hallen *et al.*, 2007, Hallen & Trail, 2008). Experiment FG7 studied the transcription profiles of germinating conidia which indicated that there are many putative secreted and wall-associated surface proteins that are differentially regulated during spore development (Seong *et al.*, 2008). In FG14 the genes that are co-regulated with DON production were studied and it was determined that *F. graminearum* has the ability to produce significantly greater quantities of DON as a result of a deletion of a specific co-regulated gene (Gardiner *et al.*, 2009a). Experiment FG2 examined gene expression under different nutrient conditions and experiments FG10 and FG11 consider the effects of DON production and identified a number of genes that were not located within the trichothecene biosynthesis cluster, but were co-regulated with the known TRI-genes (Seong *et al.*, 2009).

Interestingly, FG1 was the only experiment that looked at the global gene expression of *F. graminearum in planta* during FHB development and even here the work was on barley as a host. The infection requirements for barley by *F. graminearum* may be different to that for wheat as the spread of the fungus through the barley head does not appear to depend on the production of trichothecene toxins (Maier *et al.*, 2006). This highlights that there is a critical lack of information on the global expression of *F. graminearum* during the infection of wheat and particularly during FCR disease development.

Table 1.5: Summary of *F. graminearum* Affymetrix GeneChip experiments that are available on the plexdb database.

Experiment Accession No	Experiment Details	Reference
FG1	Time-course of FHB on barley.	(Guldener *et al*., 2006b)
FG2	*F. graminearum* expression profiles under carbon and nitrogen starvation conditions.	(Guldener *et al*., 2006b)
FG5	Transcription detection during *F. graminearum* in vitro sexual development.	(Hallen *et al*., 2007)
FG6	Transcription detection during *F. graminearum* sexual development with Cch1 calcium channel deletion.	(Hallen & Trail, 2008)
FG7	*F. graminearum* gene expression during conidia germination.	(Seong *et al*., 2008)
FG10	*F. graminearum* gene expression in response to trichodiene treatment.	(Seong *et al*., 2009)
FG11	Tri6 and Tri10 gene deletion F. *graminearum* expression.	(Seong *et al*., 2009)
FG14	DON induction media.	(Gardiner *et al*., 2009a)

Proteomic techniques have been used to complement global molecular studies of the *F. graminearum* pathogen. Proteomics of *F. graminearum in vitro* has been used to study mycotoxin production (Taylor *et al.*, 2008), proteins regulated by the fungal mating-type genes (Lee *et al.*, 2008) where the gene MAT1-2 has been linked to the regulation of many cell wall-associated proteins possibly during sexual development of *F. graminearum*. Also proteomics has been used to compare the fungal proteins produced on glucose as a carbon source compared to plant cell walls (Phalip *et al.*, 2005) where it was confirmed that many cell wall degrading enzymes (CWDE) are produced in response to contact with the host cell wall which may play a major role in virulence and pathogenicity. From these studies, sets of genes have been identified for further functional analysis based on increased production of the protein product under the relevant experimental conditions.

Metabolomics is also beginning to be used to study the *F. graminearum*-wheat interaction (Hamzehzarghani *et al.*, 2008). Metabolomics is the comparative study of primary and secondary

metabolites produced in organisms that have been grown under specific sets of environmental conditions (Stobiecki, 2009). Analysis of wheat metabolites produced in response to *Fusarium* head blight infections has identified many resistance-related (RR) compounds and some of which interestingly have unknown function (Paranidharan *et al.*, 2008, Hamzehzarghani *et al*., 2008).

F. graminearum grows as a haploid in culture, which makes it possible to investigate gene function in the laboratory by producing gene knockout mutants. Currently, there are over 27 *F. graminearum* pathogenicity and virulence genes established using gene knock out techniques (Table 1.6) including the previously discussed Tri5 genes. These genes have been collated into a publically available database called PHI-base that specializes in genes that are involved in pathogen-host interactions (www.phi-base.org) (Winnenburg *et al.*, 2006). These pathogenicity and virulence genes fall into several classes and examples of these are discussed briefly below.

GENES FOR SIGNAL TRANSDUCTION PATHWAYS

The roles of signalling pathways in pathogenicity and virulence have been well studied in phytopathogenic fungi over the past several years (Nikolaou *et al.*, 2009, Zhao *et al.*, 2007). Sensing environmental stresses such as osmotic, oxidative and cell wall stress are essential to the virulence and pathogenicity of microbes. Stress signalling is thought to have evolved specifically to suit the environment in which the microbe lives. It has been shown that a number of signalling pathways play an important role in perception and reaction to the environment during infection such as cyclic adenosine monophosphate (cAMP) and mitogen-activated protein kinase (MAPK). Phytopathogenic fungi such as *Stagonospora nodorum* and *Blumeria gruminis* utilize a cAMP-signalling pathway during early stages of infection. This pathway functions in the recognition of plant signals enabling spore germination and penetration (Solomon *et al.*, 2004, Barhoom & Sharon, 2004, Kinane *et al.*, 2000). MAPK signalling pathways have been implicated in a number of pathogenicity traits, for example, in *F. graminearum,* the MAPK gene *MGV* was shown to be a virulence factor and also involved in female fertility during sexual reproduction (Hou *et al.*, 2002). Another MAPK gene *MAP1* (*GPMK1*) was shown to be essential for pathogenicity and it was also involved in the development of perithecia and ascospores (Jenczmionka *et al.*, 2003, Urban *et al.*, 2003). Signalling genes often regulate a number of downstream processes involving many genes and it is evident from the above that they can control both pathogenicity and other developmental programs such as sexual reproduction.

Table 1.6: *F. graminearum* genes that play a role in virulence and pathogenicity during *Fusarium* infection. Table modified from www.phi-base.org.

Gene Name	PHI-base accession No	Description	Disease Name	Host
GCS1	PHI:1002	Not available	FHB	Wheat and Maize
STE7	PHI:1004	Probable MAP kinase kinase	FHB	Wheat and Tomato
FgHOG1	PHI:1005	Probable osmotic sensitive-2 protein	FHB	Wheat
HMR1	PHI:1006	Hydroxymethy-glutaryl CoA reductase	FHB	Wheat
NPS6	PHI:1007	Related to AM-toxin synthetase	FHB	Wheat
SID1	PHI:1010	Not available	FHB	Wheat
GzGPA2	PHI:1013	Probable G protein alpha subunit	FHB	Barley
GzGPB1	PHI:1015	Probable guanine nt-binding beta subunit	FHB	Barley
STE11	PHI:1016	Probable MAP kinase kinase	FHB	Wheat and Tomato
MGV1	PHI:266	MAP kinase	FHB	Wheat and Tomato
GzCPS1	PHI:304	Acyl CoA ligase-like protein	FHB	Wheat
MAP1 (related:GPMK1)	PHI:309	MAP kinase 1	FHB	Wheat and Tomato and *Arabidopsis thaliana*
GzmetE	PHI:355	Probable homoserine O-acetyltransferase	FHB	Wheat
FGL1	PHI:432	Probable triacylglycerol lipase precursor	FHB	Wheat and Maize
TRI6	PHI:439	Trichothecene biosynthesis positive transcription factor	FHB	Wheat
TRI5	PHI:44	Trichodiene synthase	FHB	Wheat and Rye
MSY1	PHI:442	Probable methionine synthase	FHB	Wheat and Maize
CBL1	PHI:443	Probable cystathionine beta-lyase	FHB	Wheat and Maize
ZIF1	PHI:444	Related to bZIP transcription factor	FHB	Wheat and Maize
NOS1	PHI:445	Probable NADH dehydrogenase	FHB	Wheat and Maize
TBL1	PHI:446	Related to nuclear receptor co-repressor	FHB	Wheat and Maize
TRI14	PHI:525	Putative trichothecene biosynthesis	FHB	Wheat
FSR1	PHI:731	Probable signal transduction scaffold protein	FHB	Barley
FBP1	PHI:733	Probable F-box protein	FHB	Barley
ARG2	PHI:743	Not available	FHB	Barley
ADE5	PHI:744	Not available	FHB	Barley
RAS2	PHI:861	Probable RAS-2 protein	FHB	Wheat and Maize

PRIMARY METABOLISM PATHWAYS

Of the known *F. graminearum* pathogenicity and virulence genes, three are involved in methionine synthesis (Figure 1.6). They are: *GzmetE* (predicted to be a homoserine O-acetyltransferase), *MSY1* (a methionine synthase) and *CBL1* (a cystathionine beta-lyase). There is little published data addressing the role of fungal methionine metabolism in regards to pathogenicity. A clue to the possible role of methionine synthesis in pathogenicity come from another plant pathogenic fungus *Botrytis cinerea* where methionine biosynthesis is important. This fungus uses methionine to produce ethylene during pathogenesis (Chague *et al.*, 2002). Interestingly, ethylene has been shown to be important in the host-pathogen interaction as it promotes senescence in host tissue (Chague *et al.*, 2006). Similarly, it has recently been shown that host ethylene signalling is necessary for full FHB and DON action in wheat (Chen *et al.*, 2009). Thus, it may be that ethylene is produced as a pathogenicity factor by *F. graminearum*. It could also be hypothesised that if the methionine biosynthesis pathway is blocked then a greater amount of homoserine may be converted to threonine. An overload of homoserine in some way may interrupt threonine biosynthesis and threonine biosynthesis has been shown to be essential for growth of *Cryptococcus neoformans* (Kingsbury & McCusker, 2008). Threonine biosynthesis pathway is conserved in yeast and fungi but it is different to that in humans (Kaplan & Flavin, 1965), thus threonine biosynthesis may be a powerful tool for developing antifungal control systems.

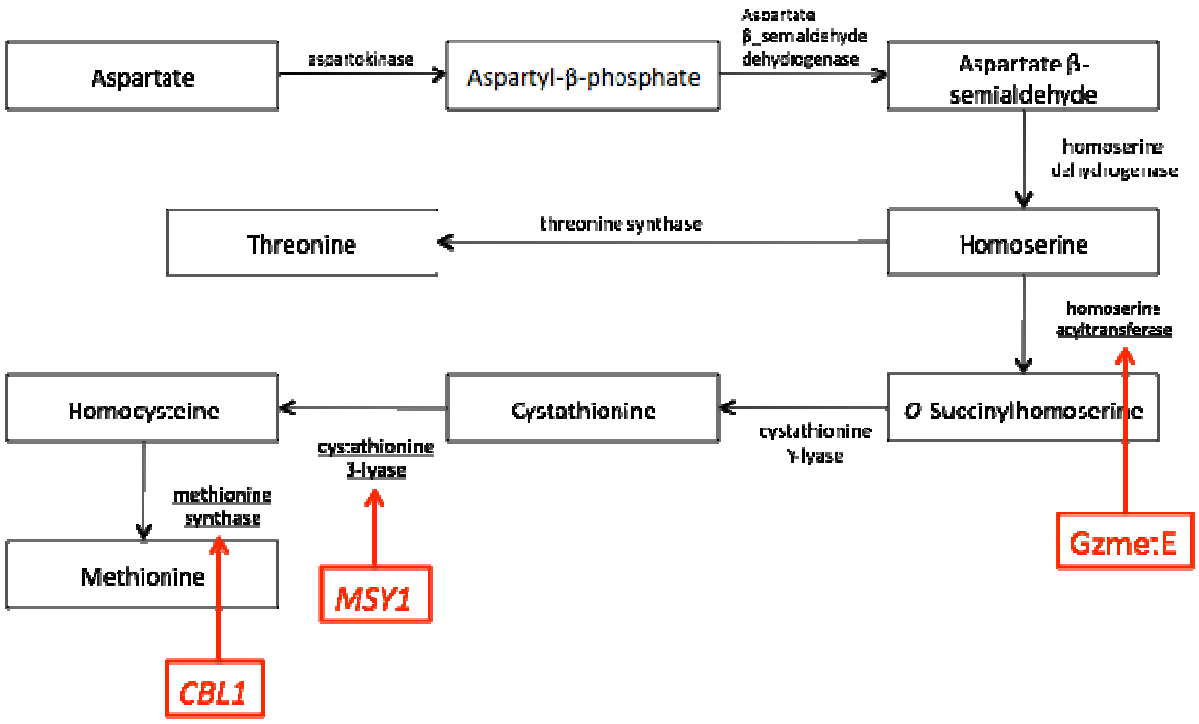

Figure 1.6: Methionine Biosynthesis Pathway. Adapted from BioCarta; GzmetE, *MSY1* and *CBL1* are indicated by using red arrows.

EXTRACELLULAR ENZYMES

Cell wall degrading enzymes (CWDEs), are produced by many pathogenic fungi at the point of entry into the host. They serve to weaken the plant cell wall and aid the mechanical invasion by the fungus (Pryce-Jones *et al.*, 1999). The array of CWDEs that are expressed can be species-specific and can include polygalacturonases (Oeser *et al.*, 2002), pectin lyases (Yakoby *et al.*, 2001), cellulases, xylanases and glucanases. Necrotrophic fungi secrete batteries of CWDEs as well as proteinases and lipases, which degrade structural macromolecules in plant cells and provide low molecular weight breakdown products that can be assimilated by the fungus. In *F. graminearum* the gene *FGL1* (Voigt *et al.*, 2005) encodes a probable triacylglycerol lipase precursor and was shown to be involved in extracellular lipolytic activity. Lipolysis may be involved in *F. graminearum* pathogenicity during FHB because it facilitates the metabolism of the oily germ of the wheat grain thus unlocking nutrient sources for the fungus that are essential for full pathogenicity.

Aims of the research project

Most of the pathogenicity genes identified in *F. graminearum* have been tested for a role in FHB in wheat or barley or disease development in another cereal host eg, maize cobs and silks. Even *Arabidopsis* flowers have been used in some assays, however, thus far there has not been a study of pathogenicity in FCR disease. In consideration of the literature discussed here it is obvious that there is a lack of knowledge at the cellular and molecular levels regarding how *F. graminearum* interacts with the wheat plant during a crown rot infection. Thus, the overall goal of this research project was to develop a better understanding of the infection processes associated with FCR on wheat and to identify fungal genes that have roles in infection of the stem and crown and compare and contrast this with FHB.

The specific aims of this PhD project were to:

1. Quantitatively and qualitatively characterise the *F. graminearum* colonisation pathway during crown rot disease of wheat.
2. Analyse gene expression changes in *F. graminearum* during crown rot disease of wheat.
3. Identify *F. graminearum* genes that may play role in virulence during either FCR or FHB.
4. Identify if known *F. graminearum* virulence factors are general or show specificity to infections of particular tissue types. For example, are genes that are important for FHB infection also important for FCR infection?

Chapter 2: Stephens, A.E., Gardiner, D.M., White, R.G., Munn, A.L., and Manners, J.M. (2008). Phases of Infection and Gene Expression of *Fusarium graminearum* During Crown Rot Disease of Wheat. Molecular Plant-Microbe Interactions 21, 1571-1581.

For supplementary data see Appendix 4.

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Phases of Infection and Gene Expression of *Fusarium graminearum* During Crown Rot Disease of Wheat

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Fusarium graminearum **causes head blight (FHB) and crown rot (CR) diseases in wheat. Compared with FHB, CR symptom development occurs slowly, usually taking 4 to 8 weeks to become visible. To characterize CR development, we used histological and real-time quantitative polymerase chain reaction analyses to assess fungal colonization during a timecourse of infection. Three distinct phases of infection were identified: i) initial spore germination with formation of a superficial hyphal mat at the inoculation point, ii) colonization of the adaxial epidermis of the outer leaf sheath and mycelial growth from the inoculation point to the crown, concomitant with a drop in fungal biomass, and iii) extensive colonization of the internal crown tissue. Fungal gene expression was examined during each phase using Affymetrix GeneChips. In total, 1,839** ***F. graminearum*** **genes were significantly upregulated, including some known FHB virulence genes (e.g.,** ***TRI5*** **and** ***TRI14*****), and 2,649 genes were significantly downregulated in planta compared with axenically cultured mycelia. Global comparisons of fungal gene expression with published data for FHB showed significant similarities between early stages of FHB and CR. These results indicate that CR disease development involves distinct phases of colonization, each of which is associated with a different fungal gene expression program.**

Additional keywords: deoxynivalenol, DON, pathogenicity.

The filamentous fungus *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* [Schw.] Petch) is one of the most important pathogens of small grain cereals. It is best known as the pathogen responsible for Fusarium head blight (FHB) disease of wheat, also referred to as head scab (Goswami and Kistler 2004). Infection of wheat heads by *F. graminearum* reduces grain yield by degrading starch granules in the kernels (Jackowiak et al. 2005). It also reduces the quality of the grain by contaminating it with harmful mycotoxins such as the trichothecene deoxynivalenol (DON), rendering it unsafe for human and livestock consumption. Because of the global importance of FHB disease, *F. graminearum* has been intensively researched. Its genome has been sequenced (Cuomo et al. 2007), and functional genomics tools have been developed, including the Affymetrix GeneChip for gene expression profiling (Guldener et al. 2006b). Mutant collections for specific genes and gene clusters also exist, but there are currently only around 26 published genes that have been shown to encode *F. graminearum* pathogenicity and virulence factors. Three of these genes (*TRI5, TRI14*, and *TRI6*) are in the well-recognized gene cluster that encodes for trichothecene production (Dyer et al. 2005; McDonald et al. 2005; Proctor et al. 1997). As expected, signaling pathways have also been shown to play a role in *F. graminearum* pathogenicity on wheat heads. One mitogen-activated protein kinase (MAPK) encoding gene, *MGV1* (Hou et al. 2002), was shown to be a virulence factor involved in female fertility during *F. graminearum* sexual reproduction. Another MAPK gene, *MAP1* (*GPMK1*) (Jenczmionka et al. 2003; Urban et al. 2003), is essential for pathogenicity and is also involved in the development of perithecia and ascospores. Considering that the plant pathogenic fungus *Magnaporthe oryzae* has over 200 pathogenicity loci (Jeon et al. 2007), it is likely that the journey to understanding *F. graminearum* pathogenicity is only just beginning.

In addition to *F. graminearum*, there are several other *Fusarium* species that cause *Fusarium* crown rot (CR) disease of wheat, such as *F. pseudograminearum*. In this study, *F. graminearum* was chosen as a model *Fusarium* pathogen for CR disease because of the extensive genomic resources available. In contrast to FHB, CR disease affects the stem base and crown of wheat plants (Mudge et al. 2006), producing necrotic lesions in these tissues. Like FHB, CR can decrease grain yield by up to 100% in Australia (Southwell et al. 2003) and 35 to 61% in North America (Smiley et al. 2005), and there is emerging evidence that CR disease may lead to contamination of wheat stubble and heads with the mycotoxin DON (Mudge et al. 2006). It is thought that the principal inoculum for CR comes from the infested stubble of the preceding year, and this can remain viable for up to two years (Burgess 2005). Through the growing season, the wheat stem base would come in physical contact with the stubble and this could facilitate the initial infection process. Farming practices such as crop rotation can decrease the severity of Fusarium diseases in wheat, but it is the consensus of the grains industry that the production of new CR-resistant wheat cultivars is highly desirable to reduce the crop losses

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*The *e*-Xtra logo stands for "electronic extra" and indicates that one supplementary figure and two supplementary tables are published online.

due to this disease. In order for this to happen, a greater understanding of the molecular interactions between the host and the pathogen is required, including the potential mechanisms involved in fungal pathogenicity and virulence.

Fungal colonization of wheat inflorescences and the associated fungal gene-expression profiles and proteomics for *F. graminearum* have been studied extensively in regard to the development of FHB disease (Guldener et al. 2006b; Kruger et al. 2002; Paper et al. 2007; Trail et al. 2003), but there is little information available about CR disease development. It has been shown that genes necessary for the biosynthesis of trichothecenes, e.g., *TRI5*, are expressed during stem infection and that, at late stages of infection of mature plants, the central stem lumen and surrounding parenchyma are extensively colonized by the fungus (Mudge et al. 2006). That study also demonstrated that disease symptoms and fungal growth appear to develop much more slowly during CR disease than FHB disease. In the present study, we report on a systematic investigation of the colonization process, using real-time quantitative polymerase chain reaction (qPCR) to estimate fungal DNA as an indirect measure of fungal biomass during infection. Three distinct phases of colonization were identified. This was followed by histological analysis of the infection process and gene expression profiling of the fungus in planta in each the three phases of infection. CR disease appears to develop in a temporally and spatially coordinated program of colonization, and the expression of fungal genes associated with specific infection stages has been observed.

RESULTS

Fungal biomass assays indicate that there are three phases of disease development during CR infection of wheat.

To investigate the CR infection process and how it relates to the slow development of CR symptoms, a timecourse of infection was carried out. Fungal biomass of *F. graminearum* was estimated as CR disease developed from the time of inoculation up to 49 days postinoculation (dpi), when symptom expression was apparent. Plant crown and stem (leaf sheath) tissue was sampled from the germinated seed to leaf 1. Real-time qPCR amplification of the DNA sequence for *F. graminearum* 18S rRNA relative to the wheat actin-binding protein DNA sequence was performed, using extracted genomic DNA from inoculated stems to estimate changes in the *F. graminearum* biomass during CR disease development (Fig. 1). This revealed three distinct phases in the colonization of wheat stem bases by *F. graminearum.* Phase 1 was a significant increase in relative fungal biomass from 0 dpi to at least 2 dpi. Phase 2 was a statistically significant decrease in fungal biomass. Finally, phase 3 was a statistically significant increase in relative biomass from 14 to 35 dpi, including a slow increase from 14 to 28 dpi followed by a rapid increase between 28 and 35 dpi, eventually reaching a plateau. An independent experiment gave similar results when fungal biomass was estimated by reverse transcriptase (RT)-qPCR of extracted total RNA amplifying the same genes but with fewer timepoints (data not shown).

Microscopic analysis of fungal colonization of wheat stems.

To further investigate the three phases of infection described above, we performed histological analysis of *F. graminearum* during CR infection. Hand sections of inoculated shoot tissue from fresh seedlings were taken at 2, 14, and 35 dpi, representing each of the three phases. Fungal tissue was visualized by staining with either the Alexa Fluor 488 conjugate of wheat germ agglutinin (WGA) that binds to lectin in fungal cell walls or with toluidine blue (Fig. 2).

At 2 dpi, the spores had germinated at the inoculation site, with germ tubes and superficial hyphae extending across the leaf-sheath surface. At this stage, there seemed to be no directional growth of germ tubes up or down the sheath, although growth around and up the trichomes was frequently observed (Fig. 2A and B).

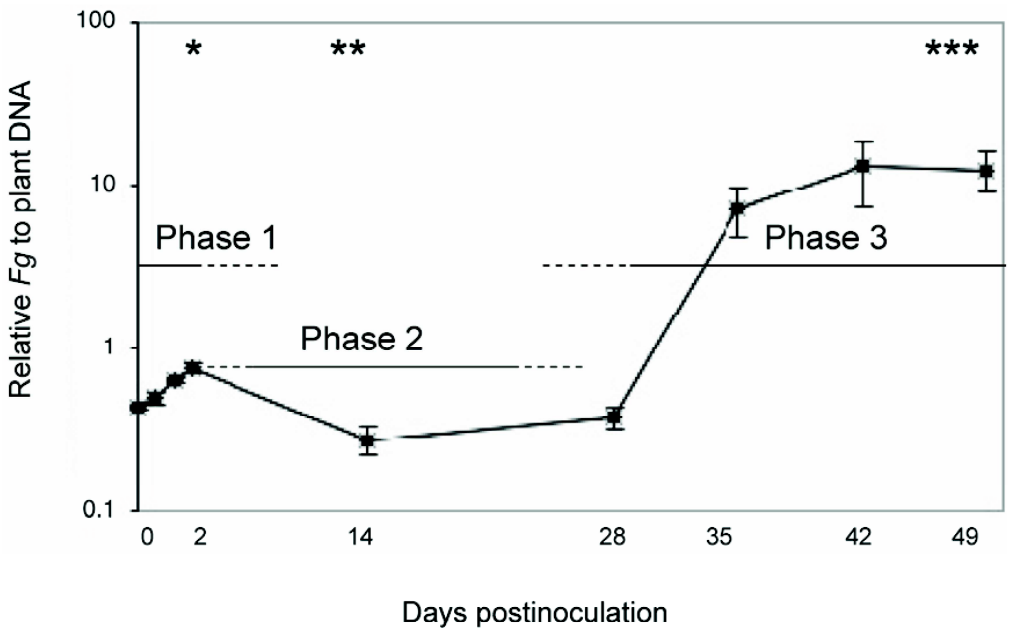


Fig. 1. Graph showing relative *Fusarium graminearum* biomass during crown rot of wheat. Fungal biomass relative to that of wheat was measured by assaying fungal 18S rDNA and wheat actin binding protein sequences by quantitative real-time polymerase chain reaction using extracted DNA as a template. All error bars are the standard error of the mean for three independent replicates. *t*-tests showed statistically significant changes in fungal biomass between the three phases. * indicates *P* value = 0.009 between 0 and 2 days postinoculation (dpi); **, *P* value = 0.008 between 2 and 14 dpi; and ***, *P* value = 0.038 between 14 and 49 dpi.

At 14 dpi, there was no evidence of mycelium at the point of inoculation. Confocal microscopy analysis confirmed the presence of *F. graminearum* hyphae inside the cells of the inner adaxial epidermis of the first leaf sheath, indicating that penetration had occurred. Interestingly, *F. graminearum* had only colonized the first leaf sheath and was growing below the point of inoculation, and most fungal colonization was observed at the base of the sheath, below the soil surface (Fig. 2C and D). At this stage, there was no visible necrotic lesion formation at the stem base.

At 35 dpi, extensive *F. graminearum* colonization was observed in the vascular tissue and pith of the crown (Fig. 2E). It appears that colonization of the interior of the wheat stem occurs at the crown after fungal colonization of the outer leaf sheath and migrates down to the base of the leaf sheath. As shown in Figure 2F, visible macroscopic symptoms started to appear at the crown of the wheat plant during this phase of colonization with the plant tissue directly surrounding the *F. graminearum* infection site becoming dark and necrotic.

Analysis of global *F. graminearum* gene expression during infection relative to cultured mycelium.

Analysis of fungal development described above indicates that *F. graminearum* has three distinct phases of infection. We were thus interested in examining whether there is differential gene expression in the fungus associated with each phase.

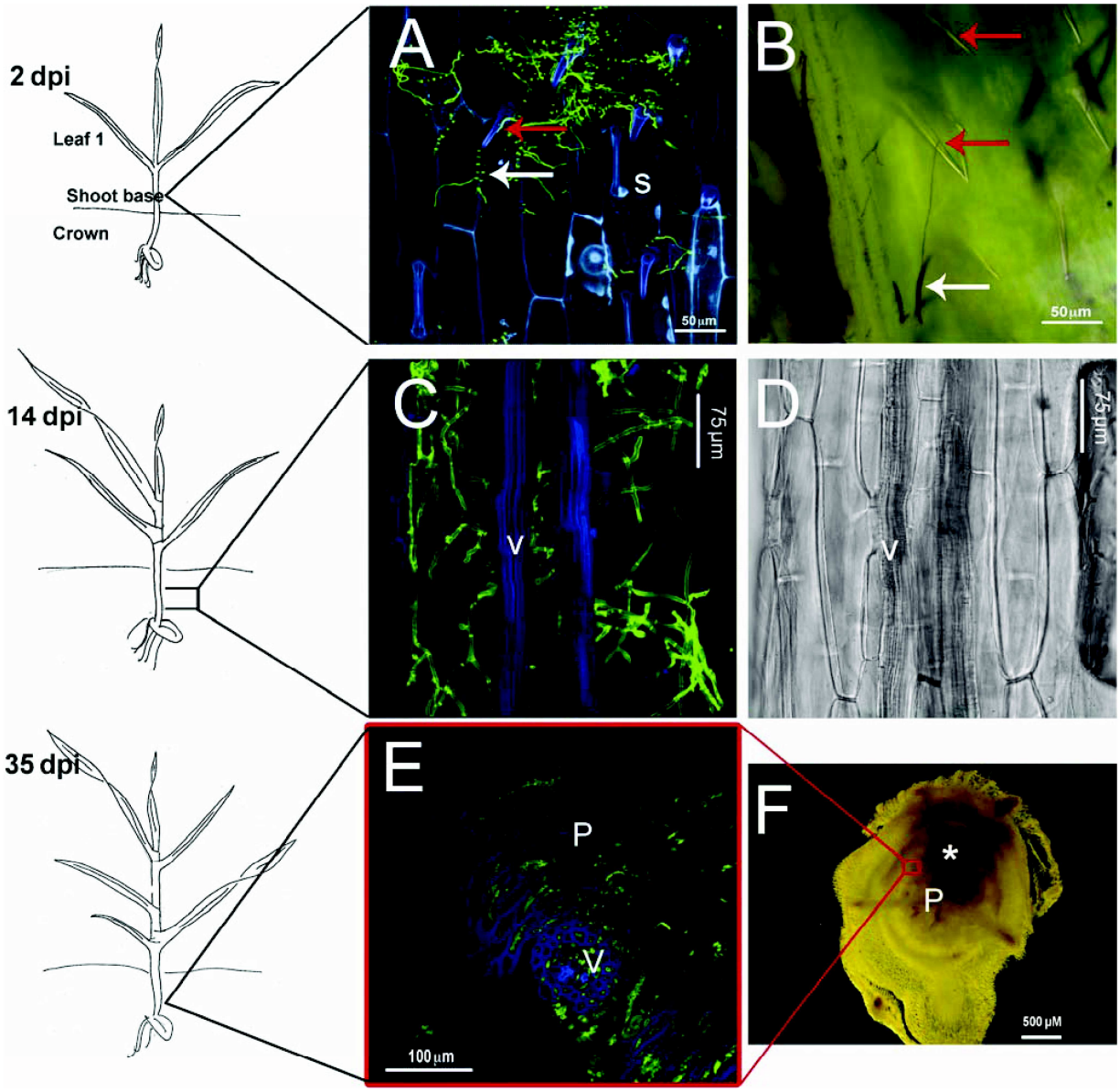


Fig. 2. Infection phases of *Fusarium graminearum* during crown rot of wheat. Plants were inoculated at the base of the shoot and sectioned by hand to visualize the extent of colonization at 2, 14, and 35 days postinoculation. **A** and **B,** Germinating spores on the leaf-sheath surface at the point of inoculation, white arrows indicate *F. graminearum* spores and red arrows show germ tube interaction with trichomes as a laser scan confocal image (A) and a bright field image stained with toluidine blue (B). **C and D,** Inner surface of leaf sheath 1 *F. graminearum* hyphae are inside epidermal cells, viewed in a confocal image (C) and a bright field view of the same field with no staining (D). **E,** Confocal image of transverse section of crown showing established colonization of pith (p) and secondary axial (v) bundles. **F,** The bright field view of the same sample as shown in E but with no staining and under less magnification; pith (p) tissue shows necrosis around the infection, center of infection is shown with an asterisk (*).

Global *F. graminearum* gene expression in planta and in vitro was analyzed using the *Fusarium* Affymetrix GeneChip (Guldener et al. 2006b). For in planta samples, wheat seedlings were inoculated with *F. graminearum* at the shoot base, and total RNA was obtained at 2, 14, and 35 dpi to correspond with phases 1, 2, and 3, respectively, of the infection process. Each timepoint consisted of at least three biological replicates taken in parallel, and each biological replicate was a pool of 18 stem bases. In order to tentatively identify *F. graminearum* genes that had been differentially expressed during growth in planta compared with axenic culture conditions, total RNA was extracted from a sample of *F. graminearum* cultured in complete defined media. This sample consisted of four biological replicates taken in parallel. Raw GeneChip data can be found in the PLEXdb database as experiment FG12.

Using GeneSpring GX 7.3 analysis software, probe sets (for convenience, hereafter termed genes) were filtered for significantly higher and lower expression levels in at least one of the three in planta timepoints (2, 14, or 35 dpi) compared with the in vitro samples (Fig. 3). The robust multiarray analysis (RMA) was used for a per chip normalization to the median to allow cross-array comparisons. In total, 1,839 genes were found to give significantly higher expression signals in planta, and 2,649 genes exhibited significantly lower signals in planta. RT-qPCR analysis of 16 genes confirmed the accuracy of GeneChip expression results; 13 of the 16 genes displayed very similar gene expression profiles between the PCR and GeneChip experiments (Supplementary Figure 1 contains data for gene names and results). Differences between the two analysis methods for three genes were at the 35-dpi stage, at which variation in expression was at its greatest. The majority of genes showing higher or lower expression in planta as compared with cultured mycelium were identified from the 2 and 14 dpi timepoints, suggesting that these early stages of CR may be critical for the ability of *F. graminearum* to successfully colonize the plant. The actual numbers of genes that showed higher or lower expression during growth in infected wheat at specific and combined infection stages are illustrated in Figure 3.

Functional classes of genes expressed in planta.

Genes showing significantly different expression in planta as compared with in vitro (up- or downregulated) were grouped according to the Munich Information Center for Protein Sequences (MIPS) FunCatDB classification of gene products from the *F. graminearum* genome (Fig. 4) and were compared with the genome as a whole to obtain a *P* value. This *P* value was used to identify which functional classes of genes were statistically significantly enriched within a list of genes. While some differentially expressed genes had potential roles identified, based on significant sequence homology to known genes, many of the genes found were of unknown function. Of the genes upregulated in planta, a significantly larger portion (59%, *P* value ≅ 0) were unclassified with respect to putative function, whereas only 25% (*P* value ≅ 1) of the genes showed lower expression and were unclassified, suggesting our understanding of the cellular processes occurring during infection is very limited.

Functional categories that were enriched in upregulated (Table 1) and downregulated (Supplementary Table 1) genes in

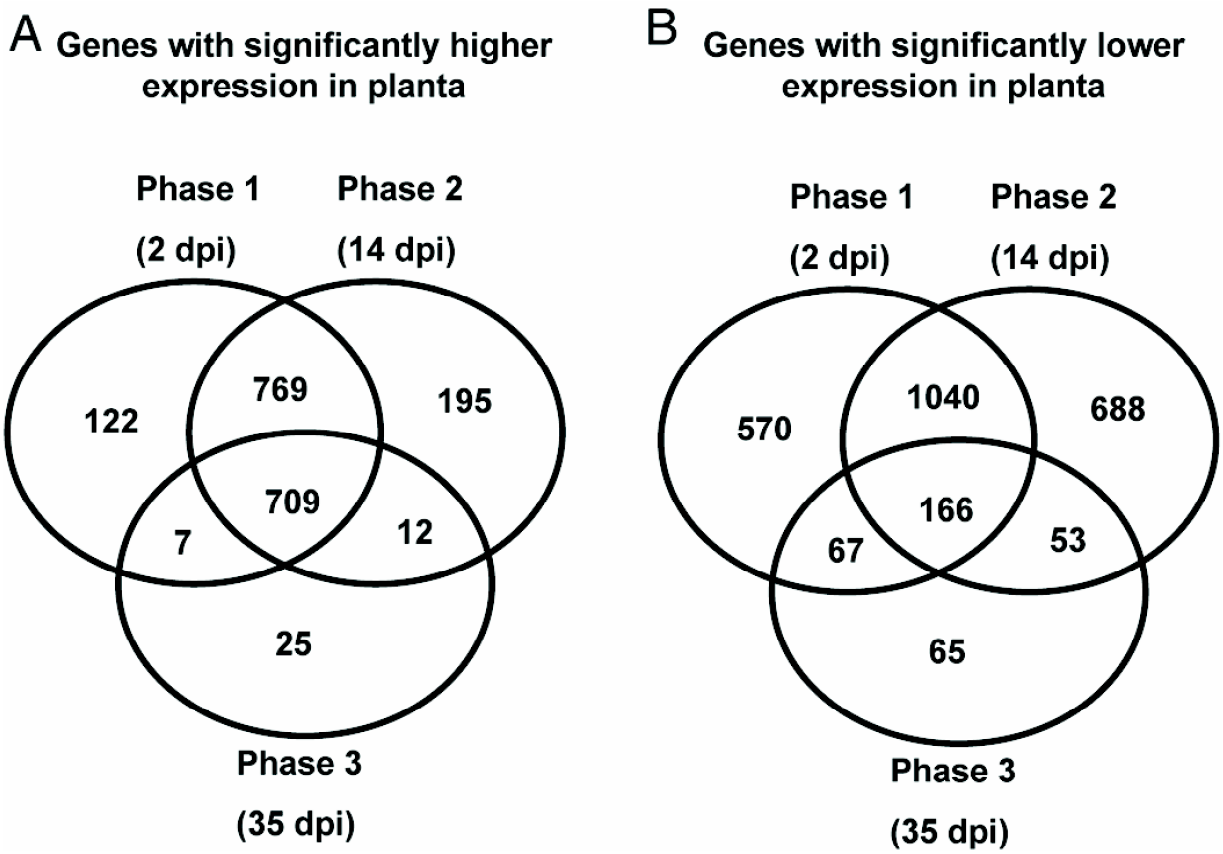
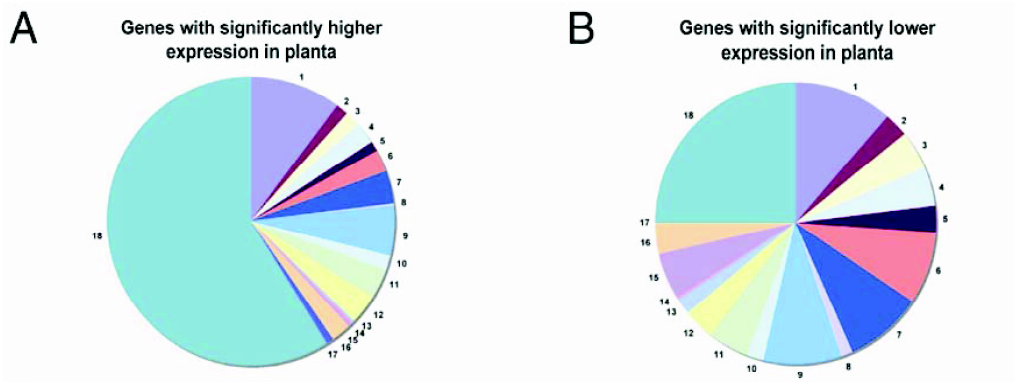


Fig. 3. Venn diagram showing the number of *Fusarium graminearum* genes with **A,** higher and **B,** lower expression during the three phases of crown rot infection (2, 14, and 35 days postinoculation) relative to axenically cultured mycelia.

planta may give an indication as to some of the important molecular processes that are taking place during the three phases of infection. For example, of the 1,947 genes downregulated at 14 dpi, genes involved in protein synthesis were significantly overrepresented compared with the rest of the genome (*P* value = 5.63×10^{-20}), suggesting that fungal growth was restricted during this phase (Gasch 2007; Holstege et al. 1998). In contrast, detoxification genes are overrepresented in the upregulated set (*P* value = 0.038) at this same timepoint. This indicates that plant defenses may be highly active in these initial phases and play a role in minimizing the relative fungal growth, consistent with the decreasing biomass observed during phase 2. Two of these upregulated detoxification genes are homologs of known virulence genes, FGSG_04580 of the *Gibberella pulicaris GPABC1* gene (90% identity), an ATP-binding cassette multidrug-resistance transporter (Fleissner et al. 2002), and FGSG_08721 of *Botrytis cinerea* BcSOD1 (77% identity), a Cu-Zn superoxide dismutase (Rolke et al. 2004). Both of these genes are being further characterized by the generation of gene knockout mutants for potential pathogenicity and virulence roles.

F. graminearum genes preferentially expressed at individual phases of infection.

Genes that had a statistically significant change in gene expression in one phase of CR infection compared with the other two phases (axenically cultured mycelia was not included in these calculations) were identified and investigated to discern important biological processes unique to any of the colonization phases. A total of 36 genes were found that had significantly different expression specifically at one of the phases of infection, with 31 showing higher expression at phase 1 and five at phase 2 (Table 2). No gene was significantly preferentially expressed in phase 3, although the error levels on expression values were larger at this timepoint because three replicates were sampled, as opposed to four replicates used for each of phases 1 and 2. Like the previous gene lists, the majority of these genes showed no homology with any genes of known function. However, among the genes showing homology was a probable glutamine synthetase (FGSG_10264) with significantly higher expression at 2 dpi, which is an indicator for nitrogen starvation in many organisms, including filamentous fungi (Palacios et al. 1978; Stephenson et al. 1997). A



C

Functional Category (MIPS Functional Catalog)	Higher Expression	Lower Expression
(1) Metabolism	10.23%	11.34%
(2) Energy	1.55%	2.96%
(3) Cell cycle and DNA processing	1.59%	4.32%
(4) Transcription	2.36%	4.30%
(5) Protein synthesis	1.27%	3.09%
(6) Protein fate (folding, modification, destination)	2.36%	8.51%
(7) Protein with binding function or cofactor requirement (structural or catalytic)	3.73%	8.81%
(8) Regulation of metabolism and protein function	0.36%	1.51%
(9) Cellular transport, transport facilities and transport routes	5.46%	8.66%
(10) Cellular communication/signal transduction mechanism	1.73%	2.06%
(11) Cell rescue, defence and virulence	3.32%	4.46%
(12) Interaction with the environment	3.27%	3.62%
(13) Cell fate	0.18%	2.09%
(14) Development (Systemic)	0.45%	0.30%
(15) Biogenesis of cellular components	0.18%	5.19%
(16) Cell type differentiation	2.27%	3.51%
(17) Organ differentiation	0.91%	0.04%
(18) Unclassified proteins	58.75%	25.05%

Fig. 4. Fungal genes with significantly altered expression in planta compared with axenically cultured mycelia grouped by their predicted function. **A,** Genes with significantly higher expression in planta, and **B,** genes that have significantly lower expression in planta. **C,** The respective number of genes in each functional group in A and B, given as a percentage of the total number of genes found to have either higher or lower expression, respectively, in planta.

condition of general nutrient starvation is suggested, because we also observed several other genes with higher expression at 2 dpi with probable roles in lipolysis. This is where lipids are broken down into fatty acids in order to utilize energy reserves. These genes include glycerol kinase (FGSG_03247), enoyl-coA hydratase (FGSG_13111), and acetyl-CoA hydrolase (FGSG_08266). A probable mannitol-1-phosphate dehydrogenase (FGSG_12827) also had a higher expression at 2 dpi, suggesting that *F. graminearum* may have been using stored mannitol as a carbohydrate source (Corina and Munday 1971).

Bioinformatic comparison of *F. graminearum* global gene expression patterns during CR and FHB on grasses.

Fusarium graminearum has the capability of causing head blight and crown rot disease in many grasses worldwide. While both diseases have been studied independently, it is widely hypothesized that the host-pathogen interaction at a molecular level will be similar in both infections, but to date, there has been little research comparing the two diseases. In previous work by Guldener and associates (2006b), the global gene expression of an American *Fusarium graminearum* isolate (PH-1) during FHB on barley was analyzed using *F. graminearum* Affymetrix GeneChip. Briefly, barley heads were infected, and samples were taken at 1, 2, 3, 4, and 6 dpi, each sample consisting of three biological replicates. The use of the Affymetrix GeneChip platform with the specified normalizations makes it possible to compare gene expression patterns from independent experiments. Therefore, to gain an overall picture of how similar the global gene expression of *F. graminearum* is during CR and FHB, sample clustering with bootstrapping was performed using the GeneSpring software. The three CR timepoints from this study and the five FHB timepoints from the previous study were used in this analysis, and the results of this experiment are graphically represented as a condition tree (Fig. 5). In general, the analysis separated the expression patterns of the two diseases and experiments, with all the CR data clustering and the FHB timepoints of 2, 3, 4, and 6 dpi also clustering. However, the results also showed that the 1 dpi FHB sample was most similar to the 2- and 14-dpi CR samples followed by the 35-dpi CR sample and clustered away from the other FHB samples. These results indicate that the infection processes of *F. graminearum*, when causing crown rot and in early stages of head blight, are significantly similar at a molecular level but are different from those associated with later stages of FHB disease development. These comparisons also suggest similarities in the infection-related gene expression patterns of the two different isolates used in these experiments and also a similarity in the processes used by *F. graminearum* to infect wheat and barley, at least at early stages of infection.

In consideration of these results, the analysis of CR and FHB together was taken a step further to identify the genes that show significantly greater expression during both the CR and FHB disease relative to that of the axenically cultured mycelium reference used in each experiment. A total of 172 genes were identified as upregulated in planta in both diseases (Supplementary Table 2), and 64 of these genes showed homology to genes of known function (Table 3). Genes involved in C-compound and carbohydrate metabolism were significantly enriched (P value = 1.38^{-03}) and were mostly depolymerases. Of these, 19 are predicted to be extracellular secreted proteins (ProtComp 6.0; Softberry, Inc., Mount Kisco, NY, U.S.A.) and most, if not all, probably act as cell wall-degrading enzymes. Homologs of enzymes that degrade cellulose, pectins, and xylans, as well as two probable cutinases were identified. Depolymerases active against other plant components were also present, with one glucoamylase that would be active against starch and two proteases. This indicates that secreted depolymerases that target polysaccharides, cutin, and protein play an integral part in *F. graminearum* pathogenicity on grasses in both CR and FHB diseases.

Also upregulated in both CR and FHB were two well-known FHB virulence genes, *TRI5* (FGSG_03537) (Proctor et al. 1995) and *TRI14* (FGSG_03543) (Dyer et al. 2005) from the trichothecene gene cluster, that determine the biosynthesis of the mycotoxin DON. This is consistent with a previous report (Mudge et al. 2006) of DON production during *F. graminearum* CR disease in wheat and the possible role it may play dur-

Table 1. Functional classes enriched for genes showing upregulated expression during crown rot (CR) compared with that of axenically cultured mycelium[a]

2 days postinoculation	*P*-value	14 days postinoculation	*P*-value	35 days postinoculation	*P*-value
99 Unclassified proteins	0	99 Unclassified proteins	0	99 Unclassified proteins	3.7E-04
01.01.05.03 Metabolism of urea (urea cycle)	0.015	01.01.05.03 Metabolism of urea (urea cycle)	0.019	20.01.07 Amino acid / amino acid derivitives transport	0.029
01.05.03 Polysaccharide metabolism	0.001	01.05.03 Polysaccharide metabolism	4.9E-05	20.03.02.02.02 Sodium driven symporter	0.035
11.06 RNA modification	0.029	01.01.06.06.01.01 Diaminopimelic acid pathways	0.025	20.09.18.07 Nonvesicular cellular import	0.045
20.01.03 C-compound and carbohydrate transport	0.028	01.01.11.01.01 Biosynthesis of alanine	0.012	32.05.05.01 Toxins	0.006
20.01.27 Drug/toxin transport	0.034	01.01.11.01.02 Degradation of alanine	0.012	34.01.03.03 Homeostasis of phosphates	0.048
20.03.02 Carrier (electrochemical potential-driven transport)	0.037	01.01.11.06 Metabolism of the D-alanine amino acid group	0.012	36.20 Plant/fungal specific systemic sensing and response	0.007
20.09.18.07 Nonvesicular cellular import	0.011	01.20.35.01 Metabolism of phenylpropanoids	0.009	40.01.05 Growth regulators / regulation of cell size	0.047
36.20 Plant/fungal specific systemic sensing and response	0.01	01.25 Extracellular polysaccharide degradation	0.042		
		16.05 Polysaccharide binding	0.04		
		20.01.27 Drug/toxin transport	0.026		
		20.03.02.03 Proton driven antiporter	0.024		
		20.09.18.07 Nonvesicular cellular import	0.009		
		32.07 Detoxification	0.038		
		36.20 Plant/fungal specific systemic sensing and response	0.011		

[a] The proportion of genes in each class in the CR upregulated data set was compared to the proportion of genes for each corresponding class across the whole genome. Gene functional categories were assigned using FunCatDB (MIPS) and P-values were calculated using the hypergeometric distribution.

ing *F. graminearum* colonization during CR of wheat. Another possible toxin biosynthetic gene was also found, NPS8 (FG00042), predicted to encode a seven-module nonribosomal peptide synthetase.

Table 2. *Fusarium graminearum* genes that are significantly differentially regulated during crown rot (CR)

Gene	Function
Genes that have significantly higher expression at 2dpi[a]	
FGSG_03970	Conserved hypothetical protein
FGSG_06082	Conserved hypothetical protein
FGSG_07488	Conserved hypothetical protein
Fg4B43_at	New open reading frame (ORF)
FGSG_01943	Conserved hypothetical protein
FGSG_11900	Conserved hypothetical protein
FGSG_08682	Conserved hypothetical protein
FGSG_07863	Conserved hypothetical protein
FGSG_05653	Related to *A. thaliana* hyp1 protein
FGSG_06682	Conserved hypothetical protein
FGSG_04933	Conserved hypothetical protein
FGSG_03715	Conserved hypothetical protein
FGSG_11296	Conserved hypothetical protein
Fg4A76_at	New ORF
FGSG_08266	Probable acetyl-CoA hydrolase
Fg3A549_at	New ORF
FGSG_08985	Probable ENA5 - Plasma membrane P-type ATPase involved in Na+ and Li+ efflux
FGSG_10264	Probable glutamine synthetase
FGSG_03247	Probable GUT1 - glycerol kinase
FGSG_12827	M1PD probable mannitol-1-phosphate dehydrogenase
FGSG_08375	Related to dicarboxylate carrier protein
FGSG_00150	Related to endo-polygalacturonase 6
FGSG_08427	Probable transcription initiation factor TFIID
FGSG_02986	Conserved hypothetical protein
FGSG_02925	Conserved hypothetical protein
Fg4A2188_at	Probable part of fg12840 putative protein [EST hit]
FGSG_07908	Reductase
FGSG_04288	Related to 26S proteasome subunit RPN4
FGSG_13111	Related to enoyl-CoA hydratase.
FGSG_07052	Related to krueppel protein.
FGSG_11900	Conserved hypothetical protein
Genes that have significantly higher expression at 14 dpi[b]	
FGSG_10653	Conserved hypothetical protein
FGSG_00729	Related to nonhistone chromosomal protein
FGSG_01161	Related to phosphatidylinositol-4-kinase
FGSG_03041	Related to alchohol dehydrogenase I -ADH1
FGSG_07213	Probable dolichol phosphate-mannose biosynthesis regulatory protein

[a] Genes that are expressed at significantly higher levels at 2 days postinoculation (dpi) than at 14 and 35 dpi.

[b] Genes that are expressed at significantly higher levels at 14 dpi than at 2 and 35 dpi.

There were also several other secreted proteins listed, such as TOX3 (FGSG_00062), which is related to KP4 killer toxin, and a gene related to a trihydrophobin precursor (FGSG_01831). Also in this list are two genes that are homologs of the *Blumeria graminis* f. sp. *hordei* Egh16 genes (FGSG_09353 and FGSG_04647) that are thought to be involved in pathogenicity during the early stages of penetration and hyphal formation of many pathogenic fungi (Grell et al. 2003).

DISCUSSION

The infection process of *F. graminearum* during CR disease of wheat was followed using a combination of biomass estimations and histological analyses. It appeared that colonization of the wheat plant by *F. graminearum* during CR proceeded in three distinct phases.

Phase 1 was a statistically significant increase in *F. graminearum* biomass in the first two days after inoculation. Confocal imaging of the inoculation point suggested that this initial increase in fungal biomass was due to the germination of spores and superficial hyphal growth on the leaf sheath abaxial surface. Interestingly, during phase 2 there was a large, statistically significant decrease in fungal biomass lasting for at least two weeks. During this period, the fungus was found to have penetrated the outer leaf sheath and to have migrated to the leaf-sheath base, where the adaxial leaf epidermis was colonized with both intracellular and intercellular hyphae. The reduction in fungal biomass during phase 2 suggests that, initially, only a small number of germ tubes successfully penetrated the leaf-sheath tissue and survived, while the hyphae remaining on the sheath surface deplete all available nutrients and subsequently perish. Phase 3 of *F. graminearum* colonization of wheat during CR involves a substantial and statistically significant increase in fungal biomass that correlates with the fungal colonization of the wheat crown parenchyma.

In FHB of wheat, the colonization pathway has been well described (Guenther and Trail 2005; Jansen et al. 2005). Briefly, after inoculation at anthesis, *F. graminearum* hyphae migrate along the epicarp to the space between the lemma and palea, where the epidermis is penetrated. This is followed by the colonization of the rachis, and movement between the florets occurs through the vascular bundles. This highlights the contrasting environments and barriers *F. graminearum* encounters during CR and FHB infections, even though they are diseases of the same host. The colonization pathway of wheat seedling tissue by *F. graminearum* during the three phases of CR seemed to correspond to specific tissue and developmental

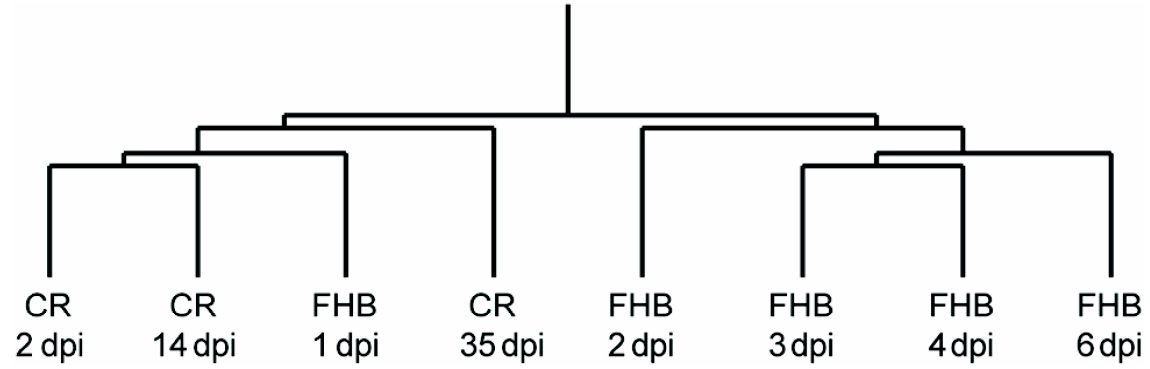


Fig. 5. Condition tree based on the similarity of *Fusarium graminearum* gene expression between each sample from Fusarium head blight (FHB) on barley (Guldener et al. 2006b) and crown rot (CR) on wheat experiment. The distance between FHB 1 day postinoculation (dpi) and CR 2 and 14 dpi is 0.24 with 100% bootstrapping confidence from 100 data sets.

stages of plant growth. During phase 2, *F. graminearum* mycelia were only observed in leaf sheath 1 below the point of inoculation and, indeed, had reached the base of the sheath below the soil surface. Like other small grain cereals, the growing point of the wheat seedling at this stage of development is below the soil surface and may provide a favorable environment for *F. graminearum* colonization because it is very rich in nutrients. During phases 1 and 2, the wheat seedling shoot is composed of layers of leaf sheaths and emerging leaves with air cavities between each layer. This stratum of physical barriers may restrict lateral penetration of *F. graminearum* into the center of the shoot, thus limiting colonization

Table 3. *Fusarium graminearum* genes whose expression is significantly upregulated in both crown rot (CR) and Fusarium head blight (FHB) and show homology to known genes

Gene	Description	Possible function
FGSG_13319	Related to transcriptional regulator atrx homolog	Transcriptional regulation
FGSG_04114	Related to adenosine deaminase	RNA metabolism
FGSG_05278	Probable amidophosphoribosyl transferase	RNA metabolism
FGSG_01290	Probable mitochondrial ribosomal protein L2 of the large subunit	RNA metabolism
FGSG_12301	Probable RNA helicase dbp2 (dead box protein)	RNA metabolism
FGSG_11064	Related to glycine-rich RNA-binding protein	RNA metabolism
FGSG_10791	Probable ATP-dependent RNA helicase DHH1	RNA metabolism
FGSG_09733	Related to 20S proteasome maturation factor	Signal transduction
FGSG_06796	Related to centractin (ARP1)	Signal transduction
FGSG_09820	Related to cysteine dioxygenase type I	Sulphur metabolism
FGSG_08126	Related to alpha glucosidase II beta subunit	Glycoprotein metabolism
FGSG_07213	Probable dolichol phosphate-mannose biosynthesis regulatory protein	Glycoprotein metabolism
FGSG_04930	Related to alpha-mannosidase 1a	Depolymerase
FGSG_02866	Possible glycosyl hydrolase	Depolymerase
FGSG_03143	Related to glycosyl hydrolase	Depolymerase
FGSG_13834	Related to bromodomain protein BDF1	Depolymerase
FGSG_11066	Related to beta-mannanase	Depolymerase
FGSG_12586	Related to beta-glucosidase	Depolymerase
FGSG_02386	Probable pectate lyase	Depolymerase
FGSG_09291	Probable pectate lyase 1	Depolymerase
FGSG_03457	Probable cutinase 1 precursor	Depolymerase
FGSG_01570	Probable cutinase precursor	Depolymerase
FGSG_09382	Probable alkaline protease (oryzin).	Depolymerase
FGD107-180	Related to xylanase (c-terminal fragment)	Depolymerase
FGSG_04768	Related to endo-1,3-beta-glucanase	Depolymerase
FGSG_06445	Probable endo-1,4-beta-xylanase	Depolymerase
FGSG_12047	Probable endo-1,4-beta-xylanase A precursor	Depolymerase
FGSG_00150	Probable NADP-dependent oxidoreductase P2	Depolymerase
FGSG_06397	Related to endoglucanase B	Depolymerase
FGSG_11488	Related to cellulose binding protein CEL	Depolymerase
FGSG_03632	Related to cellulose binding protein CEL1	Depolymerase
FGSG_03968	Related to cellulose binding protein CEL1	Depolymerase
FGSG_03194	Probable endopolygalacturonase	Depolymerase
FGSG_02202	Probable endoglucanase IV precursor	Depolymerase
FGSG_03695	Related to endoglucanase IV precursor	Depolymerase
FGSG_03315	Related to endopeptidase K	Depolymerase
FGSG_04704	Related to glucoamylase precursor	Depolymerase
FGSG_09353	Related to gEgh 16 protein	Pathogenicity associated
FGSG_04647	Probable gEgh 16 protein	Pathogenicity associated
FGSG_07832	Related to CCC1 protein (involved in calcium homeostasis)	Transporter
FGSG_04580	Probable ABC1 transport protein	Transporter
FGSG_12335	Related to DHA14-like major facilitator efflux transporter (MFS transporter)	Transporter
FGSG_03646	Related to nicotinamide mononucleotide permease	Transporter
FGSG_02580	Related to maltose permease (MalP	Transporter
FGSG_03107	Related to high affinity methionine permease	Transporter
FGSG_07227	Probable isp4 protein	Transporter
FGSG_06331	Related to zinc transporter	Transporter
FGSG_08172	Related to Cu-binding metallothionein	Oxidative stress
FGSG_07765	Related to isotrichodermin C-15 hydroxylase (cytochrome P-450 monooxygenase CYP65A1)	Oxidative stress
FGSG_02792	Related to NAD(P)H-dependent oxidoreductase	Oxidative stress
FGSG_12643	Probable sterol glucosyltransferase	Lipid metabolism
FGSG_08613	Probable OPI3 - methylene-fatty-acyl-phospholipid synthase	Lipid metabolism
FGSG_00062	TOX 3 - related to KP4 killer toxin	Small secreted protein
FGSG_08081	Related to gibberellin 20-oxidase	Gibberellin biosynthesis/ oxidative stress
FG00042	NPS8 nonribosomal peptide synthetase	Toxin biosynthesis
FGSG_03537	TRI5 trichodiene synthase	Toxin biosynthesis
FGSG_03543	TRI14 trichothecene biosynthesis gene	Toxin biosynthesis
FGSG_00656	Related to F1F0-ATP synthase subunit G	Housekeeping
FGSG_11095	Related a to carbonic anhydrase	Housekeeping
FGSG_05483	Related to ARG8- acetylornithine aminotransferase	Housekeeping
FGSG_11897	Probable UTR1 protein, associated with ferric reductase activity	Housekeeping
FGSG_00642	Related to spore coat protein SP96 precursor	Cell Surface Protein
FGSG_01831	Related to trihydrophobin precursor	Cell Surface Protein
FGSG_00449	Related to WD repeat protein IEF SSP 9502	Protein interactions

to the outer leaf sheath. Stem elongation commences during phase 3, and the elongating stem (internode) tissue provides continuity throughout the shoot, which may permit more rapid colonization of stem parenchyma. This may then enable *F. graminearum* colonization up the stem via the central stem lumen and parenchyma, toward the developing inflorescence, seen previously in mature wheat plants (Mudge et al. 2006). In the study by Mudge and associates (2006), *F. graminearum* hyphae were visualized in the stem lumen at the nodes immediately above the crown at around 56 dpi.

The delay in visible symptoms during the CR infection may be partly explained by the delayed production of DON. Studies have shown that although genes involved in the biosynthesis of DON are expressed early in CR infection, significant accumulation of the mycotoxin does not occur until 28 dpi (Mudge et al. 2006). It has been demonstrated that DON induces cell death in wheat, most probably by triggering the production of reactive oxygen species (Desmond et al. 2008) and ultimately leading to browning. The accumulation of DON in the described study coincides with the necrotrophic phase 3 of CR infection in this study, in which we see necrosis of the wheat tissue and a significant increase in fungal biomass.

Global *F. graminearum* gene expression analysis during CR infection of wheat found that 59% of significantly upregulated genes in planta were unclassified by homology in contrast to only 25% of all significantly downregulated genes, as compared with axenically cultured mycelia. This suggests that our understanding of the genes that contribute to infection is limited. A similar overrepresentation of unclassified genes was seen during the germination of conidia (Seong et al. 2008). It is understood that the cultured mycelia sample used represents a reference for only a single growth condition and phase of growth and does not comprehensively represent all genes expressed during in vitro culture.

During phase 1, genes encoding enzymes involved in the remobilization of stored nutrients as well as nutrient acquisition were frequently observed, and gene expression data suggested the processes of lipolysis, fatty acid β-oxidation, ammonium assimilation, and stored mannitol utilization were probably active. This would be expected for the initial stages of infection on the leaf-sheath surface, which is poor in nutrients. During phase 2, the reduction of biomass and upregulation of detoxification genes suggests that the pathogen is attempting to overcome host defenses. These early stages of the infection, therefore, represent the greatest opportunity for control of CR disease by preventing the extensive colonization, formation of necrotic tissue, and accumulation of DON observed at phase 3. In this study, we show that *F. graminearum* gene expression in the very early stages of FHB infection is significantly similar to those of CR. This suggests that spore adhesion, germination, penetration, and subverting initial plant defenses are comparable at a molecular level for both types of *F. graminearum* disease. Indeed, we see a number of extracellular secreted depolymerases, detoxification, and stress related genes upregulated in both CR and FHB. This also means that developing a control for CR disease may also be effective in arresting early stages of infection of FHB and vice versa.

In summary, this study gives an overview of the *F. graminearum* CR colonization pathway. We have identified three distinct phases of infection, in which fungal proliferation seems to be temporarily repressed in phase 2 before the disease really takes hold in phase 3. We have determined which tissues of the wheat plant are colonized at each phase of infection and which *F. graminearum* genes are expressed in association with pathogenicity during the infection stages. The current study serves as an excellent platform for future work identifying which genes are essential for *F. graminearum* pathogenicity during CR infection of wheat and also how host resistance may affect the stages of infection.

MATERIALS AND METHODS

Fungal strain and inoculum preparation.

For the purpose of this report the anamorph term *F. graminearum* will be used to describe the fungus. All experiments described here were conducted with the Australian *F. graminearum* isolate CS3005 (Akinsanmi et al. 2006). Macroconidia for inoculation were produced by inoculating 20 cm SNA (Spezieller Nahrstoffarmer agar) plates with a half strength potato dextrose agar (PDA) plug colonized with CS3005, then incubating at room temperature for 7 days. Spores were collected by adding 10 ml of sterile water, scraping the agar surface with a scalpel, and filtering the spore suspension through Mira cloth onto a 40 cm SNA plate and then incubating at room temperature for 7 days. Spores were washed off the SNA surface again with sterile water and the spore concentration was adjusted to 1×10^6 spores per milliliter in distilled water, and used fresh for all inoculations.

Plant growth, inoculation and harvesting technique.

For all experiments the CR and FHB susceptible bread wheat cultivar Kennedy was used. All seedlings were grown in an environmentally controlled glasshouse with day-time conditions of 24°C temperature with 60% humidity and night-time conditions of 15°C with 90% humidity. Trays of plastic seedling punnets (5 × 5 cm per punnet, 30 punnets to a tray) were filled with sterile soil mix comprising 50% sand and 50% peat (vol/vol), and two seeds were planted in each punnet. Seedlings were grown and inoculated 14 days after planting at the base of the shoot as described (Mitter et al. 2006). All seedlings were harvested and the shoot tissue from the crown to leaf 1 was excised with a pair of sharp scissors and used as a source of genetic material.

DNA extractions and *F. graminearum* biomass estimations.

Wheat seedlings were inoculated with *F. graminearum* macroconidia and harvested at 0, 0.5, 1, 2, 14, 28, 35, 42, and 49 dpi. For each timepoint three biological replicates were taken in parallel. Each biological replicate comprised a pool of 18 shoot bases. Shoot bases were ground in liquid nitrogen with a mortar and pestle and genomic DNA was extracted using a QIAGEN DNeasy plant mini kit according to the manufacturer's instructions. DNA was eluted into 100 µl of sterile water and stored at -20°C until needed. *F. graminearum* biomass was estimated indirectly using real-time quantitative PCR (RT-qPCR). PCR was performed in a total volume of 10 µl containing 5 µl of SYBR GREEN PCR master mix (Applied Biosystems, Scoresy, Victoria, Australia), 1 µl of a 3-µM mix of forward and reverse primers and 4 µl of DNA diluted 1:10 in water. Cycling conditions used were a 15 s denaturation step at 95°C then an anneal/extension step of 1 min at 60°C repeated 40 times followed by a final denaturation step. For detecting *F. graminearum* and wheat DNA, fungal 18S (Mudge et al. 2006) and wheat actin binding protein (U58278) primers (forward 5′-CGCGAGGAACAAGAT GCTGTA-3′-3′ and reverse 5′-CACGTCGATCTGCACGCC-3′), were used. Estimation of *F. graminearum* biomass was calculated according to the following equation, in which Ef is the PCR amplification efficiency and Ct is the crossing threshold.

$$\text{Rel Biomass} = \frac{\text{Ef}_{\text{Fungal}}{}^{-Ct}}{\text{Ef}_{\text{Plant}}{}^{-Ct}}$$

PCR amplification efficiencies were calculated by using the program LinRegPCR 7.5 (Ramakers et al. 2003).

Microscopic analysis of *F. graminearum* during CR infection.

Wheat plants were inoculated with *F. graminearum* macroconidia and were sampled at 2, 14, and 35 dpi. Live samples were hand sectioned at the base of the shoot and were stained with 2 μM WGA-Alexa Fluor 488 (Invitrogen, Carlsbad, CA, U.S.A.) and toluidine blue for up to 2 h. Sections were then analyzed with a light microscope, Leica SP2 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany), and a Zeiss LSM-510 META inverted confocal microscope. Excitation wavelengths used were 405 and 488 nm, with capture between 420 to 475 nm and 500 to 527 nm, respectively.

RNA extraction and microarray hybridization.

Wheat seedlings were inoculated and harvested as described above at 2, 14, and 35 dpi. For each timepoint, four biological replicate samples were taken in parallel, except for 35 dpi, for which three were taken, and each biological replicate was a pool of 18 shoot bases. Shoot bases were ground in liquid nitrogen with a mortar and pestle, and total RNA was extracted using a QIAGEN RNeasy plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, using RLT buffer and including the optional on-column DNase I digestion. Mycelial samples were grown in a 96-well plate in 100 μl of defined media per well. Spores were inoculated into the media at a final concentration of 1×10^4 spores per milliliter. The media contained 88 mM sucrose, 5 mM glutamine, 7.3 mM KH_2PO_4, 2 mM $MgSO_4 \cdot 7\ H_2O$, 6.7 mM KCl, 36 μM $FeSO_4 \cdot 7\ H_2O$, 47 μM citric acid, 32 μM $ZnSO_4 \cdot 7\ H_2O$, 1.8 μM $CuSO_4 \cdot 5H_2O$, 0.5 μM $MnSO_4 \cdot H_2O$, 1.5 μM H_3BO_3, 0.4 μM $NaMoO_4 \cdot 2\ H_2O$, 0.03% Phytagel adjusted to pH 6.5 with NaOH (Correll et al. 1987).

Labeling and hybridization of the total RNA was carried out according to the expression analysis technical manual (Affymetrix) at the Australian Genome Research Facility (Melbourne), using the *F. graminearum* Affymetrix GeneChip (Guldener et al. 2006b).

Analysis of global gene expression data.

Expression data was analyzed by importing CEL files into GeneSpring GX 7.3 (Silicon Genetics; Agilent Technologies, Palo Alto, CA, U.S.A.). Data for each chip was normalized to the median, assessed for signal quality by condition-tree clustering, and filtered on raw signal intensity of greater than 100, to determine whether gene expression was present or absent. The number of genes up- and downregulated in planta was calculated by comparing normalized expression values with axenically cultured mycelia GeneChip data of the same *F. graminearum* isolate. To do this, a Welch *t*-test with a *P* value of 0.01 and a Benjamini and Hochberg false-discovery multiple-testing correction was performed between the average of the replicate samples of axenically cultured mycelia and each of the in planta timepoints tested (2, 14, and 35 dpi). Because sufficient biological replication was used in this experiment, genes were not subjected to a fold-change threshold cutoff, any change in expression was deemed meaningful as long as it was statistically significant. *F. graminearum* genes upregulated during FHB of barley were identified using the same calculations as in CR, using the CEL files made available at the PLEXdb database (Guldener et al. 2006b). *F. graminearum* grown in complete medium (Guldener et al. 2006b) was used as the axenically cultured mycelia reference for all FHB calculations. The number of genes that showed significant differential expression in planta was calculated the same way, using the Welch *t*-test between the different CR in planta samples. To test for similarities between CR and FHB timepoints, all samples from the FHB barley experiment and all samples from the current experiment were clustered according to their global gene expression. These results were presented in a condition tree that was created using a Pearson correlation similarity measure with bootstrapping of 100 data sets, an average linkage clustering algorithm and similar branches were merged with a separation ratio of 1 and a minimum distance of 0.001. Gene functional categorization was determined using FunCatDB, with a *P* value calculated for each functional category within a list of genes to determine if that category was statistically enriched. This *P* value was calculated using the hypergeometric distribution as a cumulative probability of each single category, drawing from the population of total genes in that category found in the genome as a whole (Guldener et al. 2006a). Limitations of the software result in very small *P* values being reported as 0 and those close to 1 reported as 1.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

PLEXdb database: www.plexdb.org
MIPS FunCatDB database:
mips.gsf.de/cgi-bin/proj/funcatDB/search_advanced.pl?gene=2

Chapter 3: A *Fusarium graminearum* ABC transporter with a role in virulence during crown rot disease of wheat.

Introduction

From the gene expression studies described in Chapter 2 a small group of genes were selected for further functional analysis using gene knock-out technology. One of these genes encoded a putative ABC transporter. This gene was selected because it showed close homology to a known fungal virulence factor and was one of a number of genes involved in detoxification that were induced during crown rot disease, especially during phases 2 and 3 of the infection process. In this chapter the function of this gene in FCR and FHB diseases was investigated using gene knock out mutants.

One of the most basic systems essential to all living cells is the ability to transport material into and out of the cell through the membrane. This can be done through specialized membrane proteins that are known as transporters. One of the largest groups of transporters is the super family of ATP-binding cassette (ABC) transporters (Higgins, 1992). ABC transporters are present in archea, prokaryotes and eukaryotes and use energy derived from the hydrolysis of ATP directly to drive the active transfer of solutes across membranes (Higgins, 1992). This is different to the major facilitator family of active transporters where the proton gradient across the membrane generated by the plasma membrane H^+-ATPase is used to achieve solute transport across the membrane (reviewed by (Law *et al.*, 2008). Plasma-membrane located ABC transporters can import nutrients and environmental substrates, but they can also export harmful toxins, drugs and lipids from the cell. ABC transporters also exist on the tonoplast of eukaryotes, including fungi, and they can transport solutes from the cytoplasm to the vacuole where the solutes can be stored or broken down enzymatically (Martinoia *et al.*, 2007). Domains that are common to ABC transporters include transmembrane domains that anchor the protein in the membrane and a nucleotide-binding domain, which is the site of ATP binding and hydrolysis.

As shown in Figure 3.1 there are four features of an ABC transporter, two transmembrane domains (TM) and two ATP-binding domains (ABD). In eukaryotes, these four domains can be present on two polypeptide chains or on one polypeptide chain. The domain structure, in conjunction with what the transporter actually transports, determines the sub-family of ABC transporter that an individual member belongs to. The complex classification of ABC transporter sub-families will not be described in detail but four examples of eukaryotic exporters with different domain arrangements are shown in Figure 3.2.

Two eukaryotic ABC exporter sub-families that are commonly linked to pathogenicity and drug tolerance are PDR and MDR transporters. PDR (pleiotropic drug resistance) and MDR (multidrug resistance) are transporters that have the ability to export more that one compound and are both synthesised as one polypeptide chain but PDR proteins are distinguished by the ATP-binding domain being positioned closer to the N terminus of the protein (Brule & Smart, 2002).

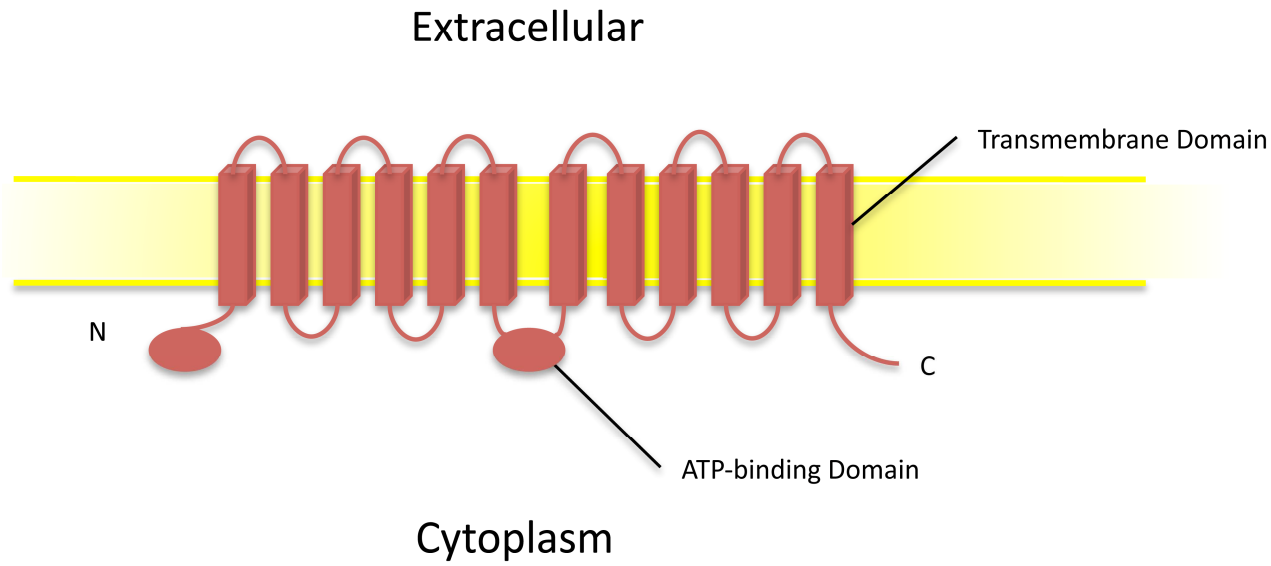

Figure 3.1: Architecture of a typical ABC transporter. There are two transmembrane domains composed of typically 6 TM-domains. There are also two ATP-binding domains that can also be referred to as nucleotide-binding domains.

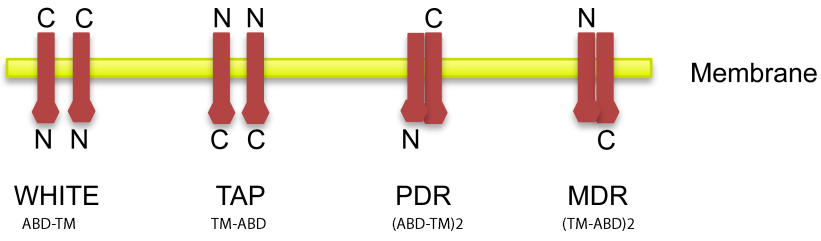

Figure 3.2: Four eukaryotic ABC exporters with different domain arrangements. WHITE and TAP are formed from two polypeptide chains and PDR and MDR are formed from one polypeptide chain. Figure modified from http://www1.pasteur.fr.

Table 3.1: Examples of ABC transporter genes from phytopathogenic fungi where a role in virulence has been demonstrated.

Fungus	Gene Accession (Gene name)	Plant Host	Substrate	Reference
Magnaporthe grisea	AF032443 (ABC1)	Rice	Unknown	(Urban *et al.*, 1999)
Botrytis cinerea	AJ006217 (BcatrB)	*Arabidopsis*	Camalexin	(Stefanato *et al.*, 2009)
Gibberella pulicaris	AJ306607 (Gpabc1)	Potato	Rishitin	(Fleissner *et al.*, 2002)
Mycosphaerella graminicola	AJ243113 (MgAtr4)	Wheat	Azole fungicides, Steroids Diacetoxyscirpenol, Rhodamine 6G	(Stergiopoulos *et al.*, 2003) (Zwiers *et al.*, 2003)
Fusarium culmorum	DQ489315 (FcABC1)	Wheat	Unknown	(Skov *et al.*, 2004)

Many studies have shown that ABC transporters, particularly those of the MDR and PDR classes, are important to the resistance of human fungal pathogens to anti-mycotic agents (Niimi *et al.*, 2004). Similarly, ABC transporters have been shown to confer resistance to fungicides in fungal plant pathogens (Zwiers *et al.*, 2003). In addition to influencing the success of fungal pathogens when challenged by fungicide treatment, ABC transport proteins have also have been shown to play an important role in virulence in several plant pathogenic fungi (Table 3.1). It is suggested that these ABC transporters may aid fungal virulence by either exporting plant-derived compounds that would otherwise be toxic to the cell or export factors that aid the infection process. These fungal pathogens include several pathogens of wheat and other monocots. For example, *Mycosphaerella graminicola* (MgAtr4) (Stergiopoulos *et al.*, 2003) and *Fusarium culmorum* (FcABC1) (Skov *et al.*, 2004) are wheat pathogens in addition to *Magnaporthe grisea* (ABC1) which is the cause of rice blast disease on rice and barley (Urban *et al.*, 1999). As a defence response many plants produce antifungal compounds called either phytoalexins as reviewed in (Gonzalez-Lamothe *et al.*, 2009). Phytoalexins are antimicrobial compounds that are produced by the plant as a defence response to infection. Examples of phytoalexins are rishitin in potato (Tomiyama *et al.*, 1968), scopoletin from tobacco (Chong *et al.*, 2002, Valle *et al.*, 1997), camalexin from *Arabidopsis* (Glawischnig, 2007) and momilactone A from rice (Cartwright *et al.*, 1981). In some pathogens, e.g. *G. pulicaris* and *B. cinerea*, a direct involvement of ABC transporters in the export of phytoalexins rishitin and camalexin, respectively, has been demonstrated (Table 3.1). However, phytoalexins from wheat have not yet been clearly defined. It

is known that wheat produces a range of low molecular weight secondary metabolites that may function as phytoalexins (Moraes *et al.*, 2008). Therefore, it is likely that *F. graminearum* would encounter such defences during infection of the stem and crown.

Results

SELECTION AND PRELIMINARY TESTING OF POTENTIAL VIRULENCE GENES OF *F. GRAMINEARUM*

Five *F. graminearum* genes were selected for further functional analysis based on their transcription profiles studied in Chapter 2. These genes are listed in Table 3.2 and include a probable superoxide dismutase, trypsin precursor, ABC transport protein, trihydrophobin precursor, a probable Egh 16 protein and one conserved hypothetical proteins. Gene knock-out vectors were prepared for each gene (Appendix 1) and putative gene knock out mutants were identified using PCR assays to test for homologous recombination events. Mutant colonies could not be obtained for FGSG_04647 and the few mutant colonies produced for the trihydrophobin precursor FGSG_01831 were shown to be only ectopic in nature. Therefore, because of the constraints, studies of FGSG_04647 and FGSG_01831 were abandoned. Representative knock-out mutants of the other genes were single spored and then tested in small scale experiments for pathogenicity on wheat (Appendix 1, Figure A1 and A2). Only two mutant types showed a potential change in disease phenotype in these assays. Mutants for a probable ABC transporter and a probable superoxide dismutase are described in detail in this and the subsequent chapter, respectively, including larger scale and more robust assays of function in virulence in FCR and FHB.

Table 3.2: List of *F. graminearum* genes selected for functional analysis. Experiments completed are indicated by a check symbol (√).

Gene	**Vector construction**	**Mutant KO creation**	**Phenotypic test**
FGSG_08721: probable superoxide dismutase	√	√	√
FGSG_11164: probable trypsin precursor	√	√	√
FGSG_04647: probable Egh 16 protein	√	-	-
FGSG_11296: conserved hypothetical protein	√	√	√
FGSG_04580: probable ABC1 transport protein	√	√	√
FGSG_01831: related to trihydrophobin precursor	√	-	-

PHYLOGENETIC AND EXPRESSION ANALYSIS OF *FGABC1*

By homology there are many genes (>70) within the *F. graminearum* genome that are predicted to encode proteins homologous to ABC transporter-like proteins. In Chapter 2, a putative ABC transporter (*FGSG_04580*) was identified as being interesting because its gene transcription was significantly higher during both FCR and FHB of wheat. Here, this gene will be referred to as *FgABC1*. This gene encodes a 1,489 amino acid protein that was predicted to be an exporter molecule within the pleiotropic drug resistance (PDR) subfamily of ABC transporters. The PDR-type ABC transporters have the ability to export several structurally unrelated compounds (Niimi *et al*., 2004, Saunders & Rank, 1982). *FgABC1* is located towards one end of chromosome 2 in an area of high SNP density (Figure 3.3) and this suggests that this gene may be evolving more quickly compared to genes in other parts of the fungal genome with lower SNP density.

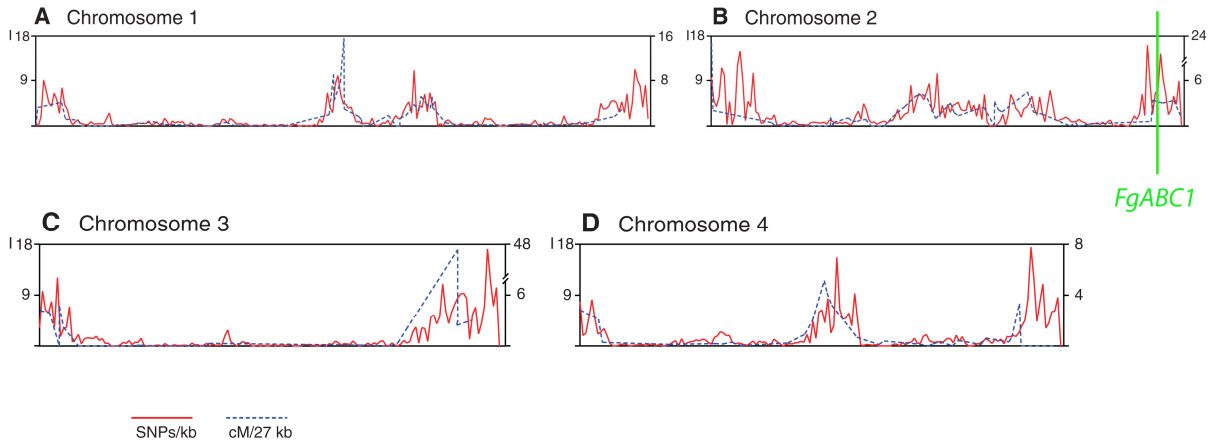

Figure 3.3: Chromosomes 1 to 4 of *F. graminearum* are represented as graphs (A) to (D) respectively. SNP density is shown with a red line and recombination rate is shown as a blue dash. SNPs are referenced to the left y axis as the number of SNPs per kb of high-quality aligned bases and recombination rates are referenced to the right y axis as cM/27kb. A total of 10,495 SNPs were found from comparing two *F. graminearum* strains PH-1 and GZ3639. Position of *FgABC1* on chromosome 2 is indicated with a vertical green line. Figure is modified from (Cuomo et al., 2007).

Global gene expression analysis during FCR (Chapter 2) and FHB (Guldener *et al*., 2006b) revealed that the transcript profile for *FgABC1* was similar during both FCR and FHB. That is, the transcript level of *FgABC1* was significantly higher *in planta* compared to vegetative mycelia and increased

over the course of the respective infection (Figure 3.4). As for most aspects of the FCR and FHB disease development, the time-span over which these changes in gene expression were observed was much shorter for FHB than for FCR.

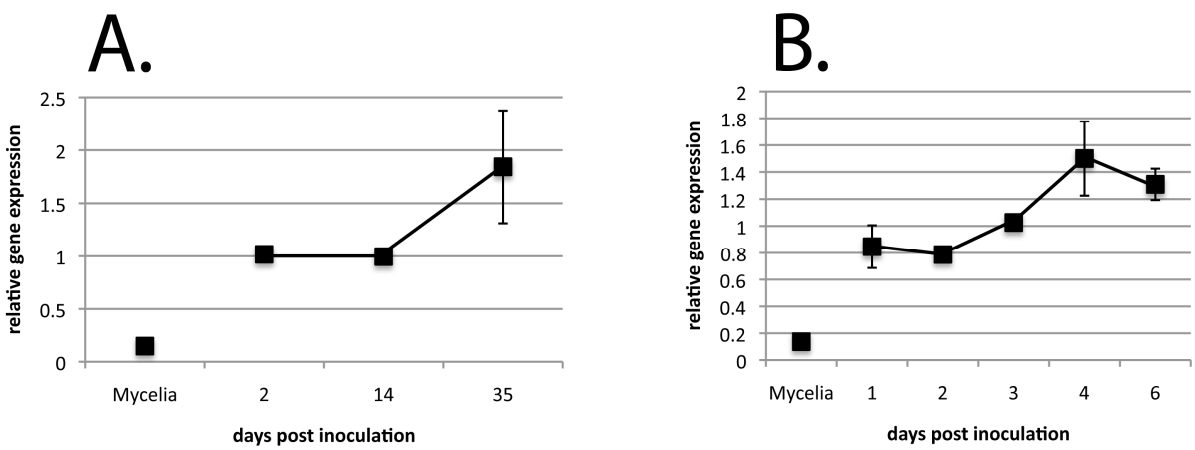

Figure 3.4: *FgABC1* gene expression (A) during FCR of wheat (Chapter 2). (B) during FHB of barley (Guldener *et al.,* 2006a). Error bars are the standard error of the mean for three to four biological replicates. Error bars for mycelia data-points are too small to be visible.

Phylogeny analyses of fungal ABC transporters showed a close relationship between *FgABC1* and *GPABC1* from *Gibberella pulicaris* and *FcABC1* from *Fusarium culmorum* as shown in Figure 3.6. There are five other *F. graminearum* ABC-transporter encoding genes present in this analysis but these show greater distance from *FgABC1*. The deduced protein sequence of the *FgABC1* gene product was 90% identical to the product of the known virulence gene *GPABC1* (Fleissner *et al.*, 2002) (Figure 3.5) and 96% identical to the product of the partial cDNA clone *FcABC1* that has been studied in regard to virulence during FHB of wheat (Skov *et al.*, 2004). In *G. pulicaris*, isolates that lacked *GPABC1* could not proliferate on slices of potato at the same rate as their wild type counterparts and it was shown that the mutants were sensitive to the potato phytoalexin rishitin. In *Fusarium culmorum*, isolates that lacked *FcABC1* showed significantly less FHB symptoms after inoculation of wheat cv. Remus, but there was no statistically significant difference in FHB symptoms on three other wheat varieties (cv. Nandu, Hope and Frontana). Because the *FcABC1* mutant varied in its ability to cause FHB between wheat cultivars it is possible that this gene is either linked to the tolerance of a plant-derived compound that varies in concentration

across wheat varieties, or to the export of a fungal specificity-determining compound that varies in activity towards different cultivars of wheat. Although there have been no wheat-derived phytoalexins indentified to date, it is hypothesised that *FgABC1* could be linked to virulence or pathogenicity of *F. graminearum* by exporting a wheat phytoalexin or other toxic compound out of the fungal cell. To test this hypothesis, deletion mutants of *F. graminearum* were created and phenotyped during both FCR and FHB of wheat.

GENERATION AND VALIDATION OF *FGABC1* DELETION MUTANTS OF *F. GRAMINEARUM*

In order to test if *FgABC1* plays a role in pathogenicity or virulence during FCR or FHB, gene deletion mutants were generated. The split marker protocol (de Hoogt *et al.*, 2000, Fairhead *et al.*, 1996) was used to produce the knock out constructs and the protoplast transformation protocol was used to create mutant isolates (Desmond *et al*., 2008b). The transformation produced 10 colonies, five of these colonies were screened by PCR, of which three were shown to be homologous recombinants and this was confirmed by Southern blot analysis (Figure 3.7). In this analysis the predicted 2.3 kb reduction in size of an *Xba*1 fragment was observed at the locus following vector integration. All three independent *FgABC1* mutants were tested visually for conidiation, perithecia and ascospore formation, which showed that there were no defects observed during these reproductive stages. To confirm that the three *FgABC1* mutants did not inherit any vegetative growth defects during the transformation process, growth rates of the mutants on minimal and complete media were quantitatively assessed. As shown in Figure 3.8, there were no significant differences in growth between the wild type and the mutants on either defined minimal or complete (V8 juice) nutrient agar medium.

Figure 3.5: ClustalW2 amino acid sequence alignment showing 90% identity between FgABC1 and GPABC1 (NCBI accession: CAC40023) gene products.

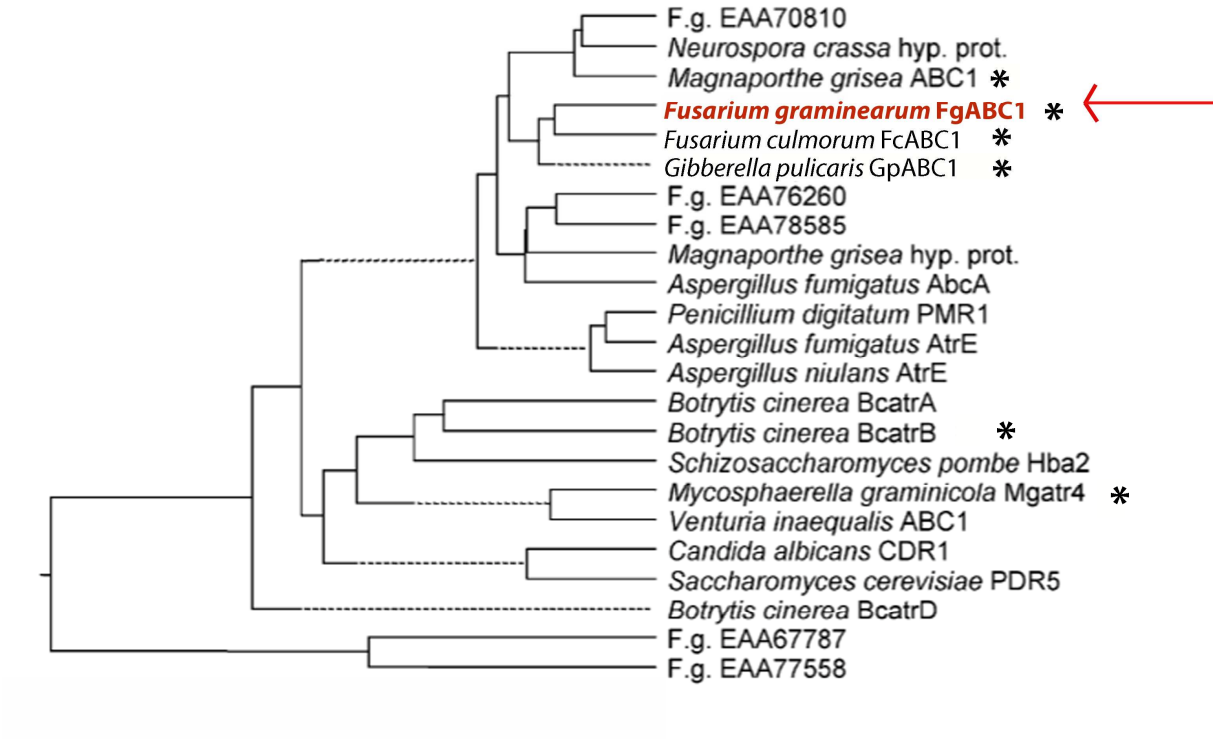

Figure 3.6: Phylogeny tree showing relationship of fungal ABC transporter proteins. FgABC1 groups with known virulence factors FcABC1 and GpABC1. Figure modified from (Skov *et al*., 2004). * indicates genes already known to have a role in virulence.

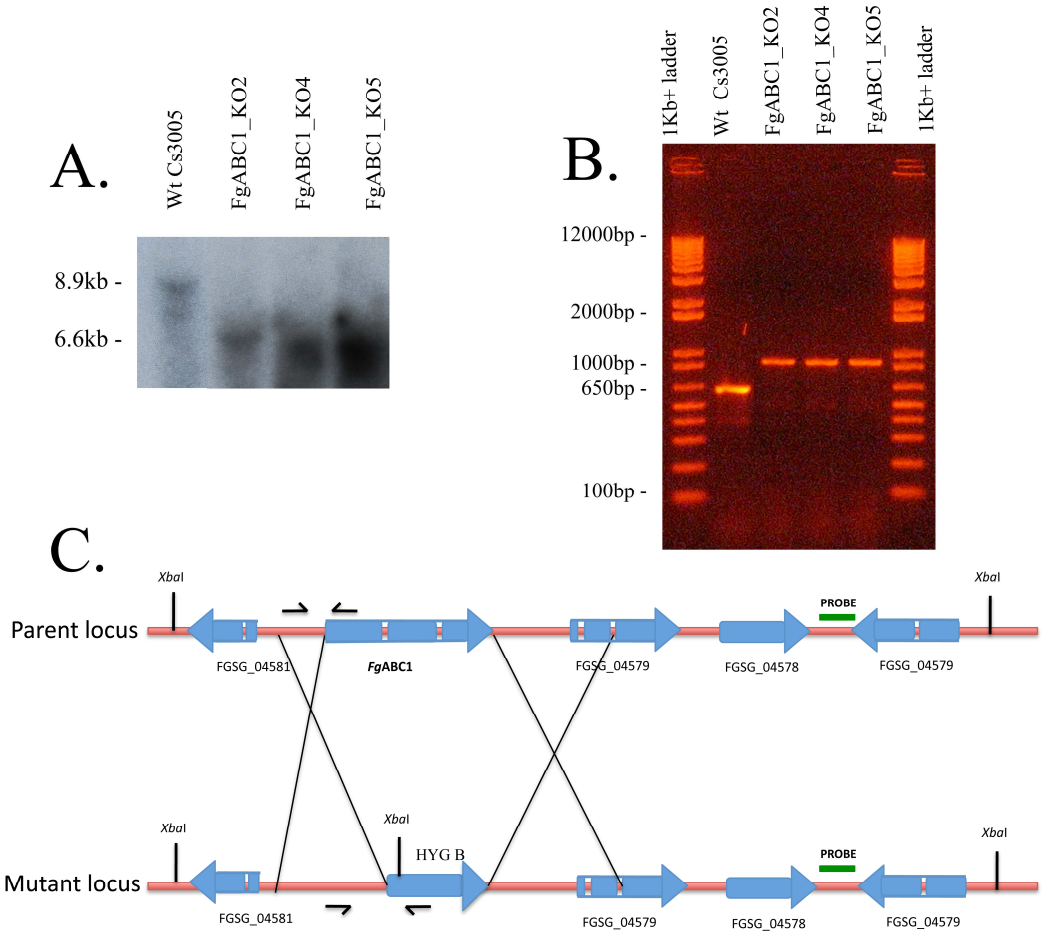

Figure 3.7: Homologous recombination validation of *FgABC1* mutants. (A) Southern analysis where genomic DNA was cut with *Xba*I and hybridization was with the probe whose location is shown in C. Wild type genomic fragment of 8.9 Kb and mutant genomic fragment of 6.6 Kb indicate gene replacement. Contrast has been adjusted on individual lanes to increased clarity of the DNA band. (B) Amplification of *F. graminearum* wild type and *FgABC1* mutants. Multiplex PCR was used to show homologs recombination had resulted in the deletion of the complete *FgABC1* open reading frame in the *FgABC1* mutants. PCR fragments of 656 bp and 844 bp were amplified from the wild type and mutant genomic DNA respectively. An ectopic recombination would show both bands. (C) Schematic representation of the recombination event including probe, primer and genetically identical cassette flanking region locations.

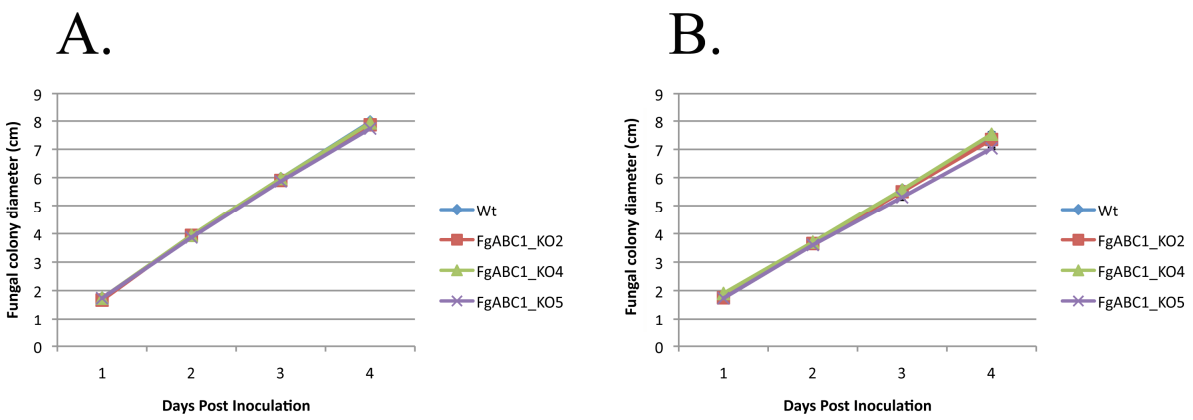

Figure 3.8: Vegetative growth of *F. graminearum* Wt (CS3005), FgABC1_KO2, FgABC1_KO4 and FgABC1_KO5 on (A) defined minimal media and (B) complete media (V8 juice agar). All error bars are the standard error of the mean for three independent biological replicates.

TESTING THE ROLE OF *FGABC1* DURING FCR DISEASE OF WHEAT

After confirming the three independent *FgABC1* mutants did not have any growth defects *in vitro*, the isolates were then tested in a crown rot bioassay to test whether *FgABC1* has a role in *F. graminearum* virulence or pathogenicity during FCR. Experiments were conducted under containment conditions approved by the Office of the Gene Technology Regulator, Australia. To do this, wheat seedlings were drop inoculated with conidia at the stem base and crown rot disease severity was assessed at 35 dpi. The calculated crown rot severity index showed that there was a decrease in crown rot symptoms between the wild type and the *FgABC1* mutants, two of which were highly statistically significant (*P-value* = < 0.01) (Figure 3.9). The mutant FgABC1_KO2 had a mean disease index half that of the wild type strain, but the test for this strain was more variable and had a *P-value* of 0.11 when compared to wild type. These results, in general, indicate that this ATP-binding cassette transporter most probably plays a significant role in the development of the typical necrotic lesions of a FCR infection in wheat.

To assess the degree of colonisation of the wheat stem base, fungal biomass was estimated using real time qPCR analysis of DNA as detailed in Chapter 2. Three biological replicates were created for each isolate by pooling the tissue from 8-10 inoculated stem bases. Even though the mean fungal biomass for FgABC1_KO4 and KO5 mutants were lower than that of the wild type, these results indicated that there was no statistically significant difference in the amount of fungal

biomass accumulated between the *F. graminearum* wild type and FgABC1_KO4 and FgABC1_KO5. The principle problem with the experiment was the high degree of variability between the three wild type biological replicates. Stem samples from *Fg*ABC1_KO2 inoculation are not included due to a technical problem with the PCR assays resulting from an unknown source of contamination in the extracted DNA (Table 3.3).

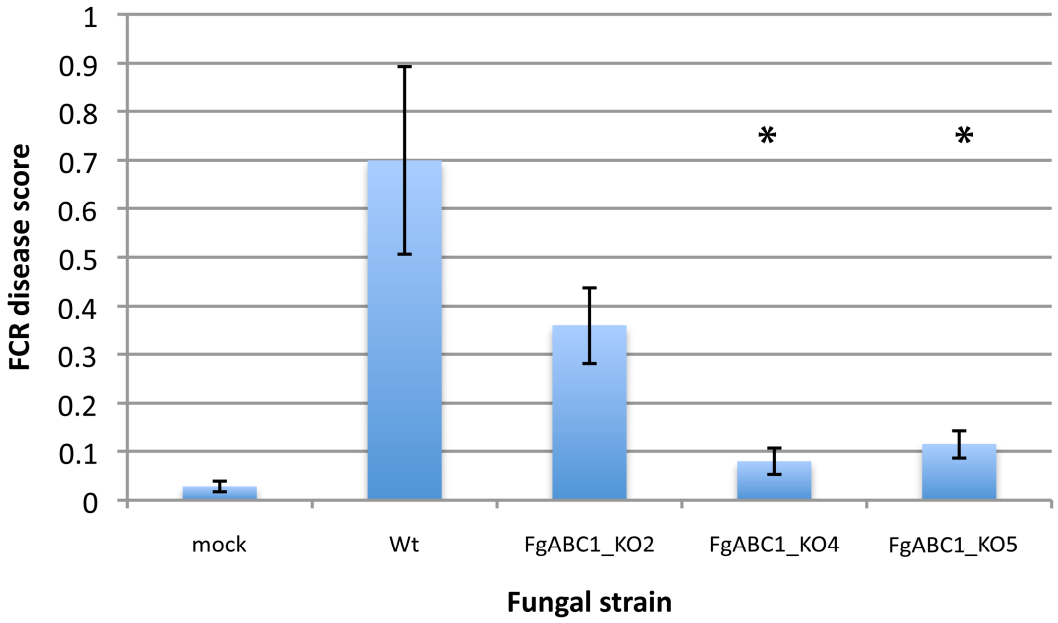

Figure 3.9: FCR disease score of wheat infected with mock (water), wild type (CS3005) *F. graminearum* and the three independent *FgABC1* mutants. Statistically significant *P-values* * are as follows FgABC1_KO4 = 0.003 and FgABC1_KO5 = 0.006 while the *P-value* for FgABC1_KO2 was 0.11. All error bars are the standard error of the mean for 25-30 independent biological replicates.

Table 3.3: *F. graminearum* wild type and mutant biomass accumulation during FCR infection of wheat. Variability shown as standard error.

	Fungal biomass	Standard error
Wild type	6.8	1.9
FgABC1_KO2	na	na
FgABC1_KO4	3.1	0.1
FgABC1_KO5	5.9	0.2

TESTING THE ROLE OF *FGABC1* DURING FHB OF WHEAT

Global analysis of *F. graminearum* gene expression in the very early stages of FHB and FCR disease development on cereals was statistically similar at the molecular level during the early stages of infection (Chapter 2). This suggests that the fungus may require similar processes for successful infection at both the inflorescence and stem base for FHB and FCR respectively in the initial stage but different at latter stages. Therefore, all three *FgABC1* mutant isolates were tested for virulence and pathogenicity during FHB. To do this, wheat heads were point inoculated at mid-anthesis as described by (Gardiner *et al*., 2009b) and disease severity was assessed 14 dpi by measuring the length of the necrotic lesion on the rachis. The results show that there was no significant difference in development of visible symptoms on wheat heads inoculated with *FgABC1* mutants compared to the wild type (Figure 3.10). This suggests that *FgABC1* does not play an important role in the virulence of *F. graminearum* during FHB in this cultivar of wheat (Kennedy).

The lack of statistical significance between the wild type and mutants were confirmed by a smaller independent experiment where wheat heads were point inoculated in the same manner and the degree of bleaching was assessed. In this latter experiment, on average the FHB infection caused by the wild type induced 64.1% of the wheat florets to exhibit symptoms, FgABC1_KO2 = 63.6%, FgABC1_KO4 = 29.9% and FgABC1_KO5 = 63.5%.

FUNCTIONAL ANALYSIS OF *FGABC1* TO IDENTIFY CANDIDATE TRANSPORT COMPOUNDS

After establishing that *FgABC1* was linked to disease symptom development during FCR of wheat it was considered important to test how *FgABC1* may be functioning. Transporters within the ABC family have been reported to transport a range of biological and inorganic compounds. As indicated earlier, one potential role for FgABC1 was that it might export defensive compounds produced by wheat. To gain a better understanding of what type of compounds this particular transporter may be involved in moving, the growth of the wild type and mutant isolates were measured when grown in media containing different compounds. To do this 3 different phenotype array plates (Biolog PM21B, PM22A and PM24A) were inoculated with *F. graminearum* wild type and mutant conidia spores. Optical density (OD) readings were then taken each day for three days. Each array plate contained 24 different compounds, making a total of 72 compounds tested in total, and 4 different concentrations were tested for each compound.

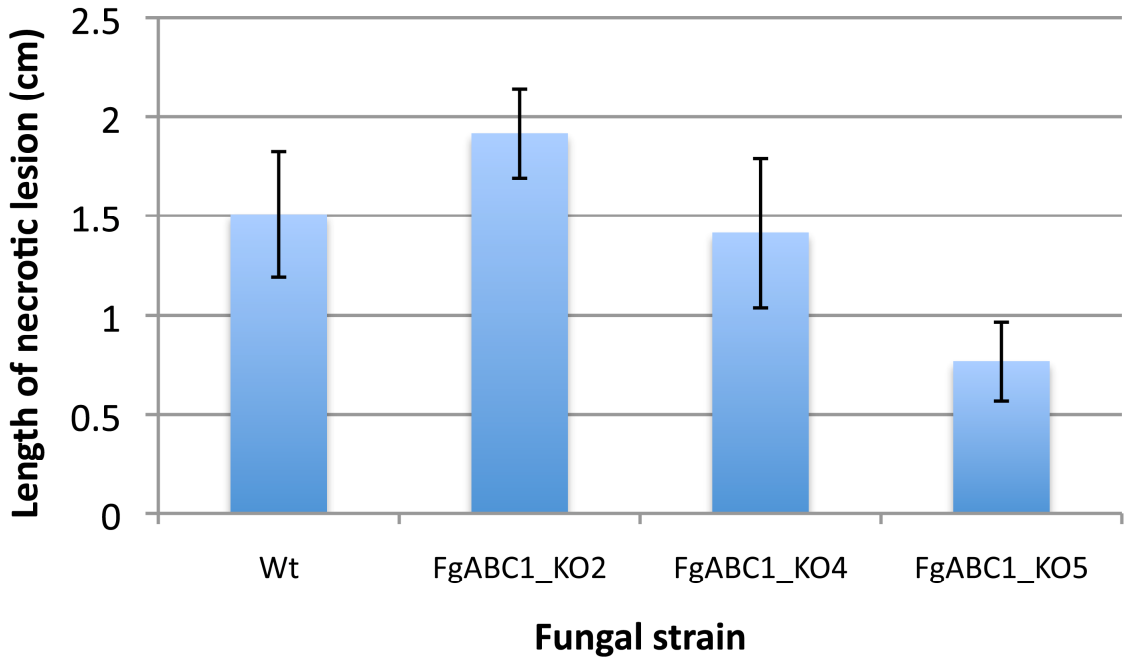

Figure 3.10: *F. graminearum* pathogenicity during FHB on wheat showing no statistically significant difference in pathogenicity of FgABC1_KO2, FgABC1_KO4, FgABC1_KO5 and the wild type. All error bars are the standard error of the mean for 13-15 independent biological replicates.

The complete list of compounds tested on these array plates is shown in Appendix 2, but they included many organic and inorganic compounds with known antimicrobial activity. Results showed that at 3 dpi there were 3 compounds (salicylanilide, benalaxyl and diamide) that showed a decrease in mutant growth compared to the wild type isolate in at least one of the concentrations tested (Figure 3.11). Due to experimental resource constraints only 2 biological replicates were tested per sample, therefore both biological data-points are plotted in all the graphs shown. Growth of FgABC1_KO4 in salicylanilide was lower compared to the wild type for both biological replicates over all four of the concentrations (50, 150, 450 and 1350 µg/mL in Figure 3.11A). Mutant growth in media containing benalaxyl was not affected at the lowest concentration (45µg/mL) but was lower in the three higher concentrations (135, 405 and 1215 µg/mL Figure 3.11B). Mutant growth in diamide was only inhibited at the highest concentration of 500 µg/mL, at this concentration the mutant showed an average of a 6.4 fold decrease in growth compared to the wild type (Figure 3.11C). All three of these compounds possess antifungal activity and have been

used as commercial fungicides. These compounds are structurally diverse indicating that the transporter may have a broad substrate range.

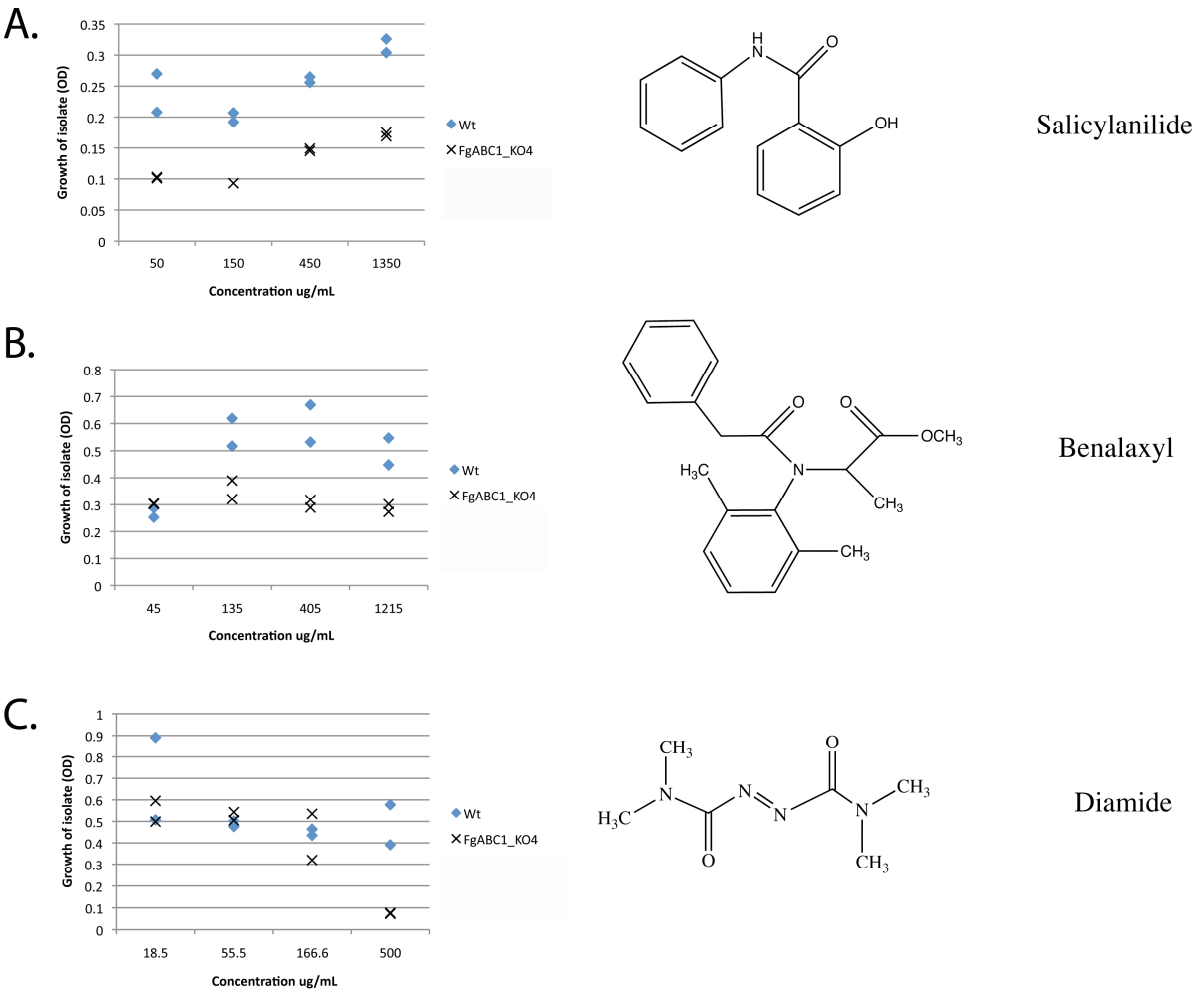

Figure 3.11: Growth of *FgABC1* mutant and wild type in the presence of three compounds at increasing concentrations (A) Salicylanilide (B) Benalaxyl and (C) Diamide. Growth was calculated as an optical density reading. For each isolate there were two independent biological replicates and both data-points are displayed. Wild type data-points are marked as a blue diamond and mutant isolate data-points are marked as a black cross. Where a single data point is shown both replicates showed identical readings.

Discussion

ABC transporters are responsible for the export of many compounds that would otherwise be toxic to the fungal cell and thus can act as virulence factors during host-pathogen interactions. The fungal ABC transport system is large and complex with over 70 *F. graminearum* genes showing homology to ABC transporter-like genes. Because many ABC transporters have the ability to transport multiple types of compounds and there are a large number of ABC transporter genes present in the genome, one may expect there to be a large degree of redundancy for their function. Interestingly, this was not the case with *FgABC1*, as it was shown to be necessary for the virulence of *F. graminearum* in the development of FCR disease symptoms in wheat.

HOW DOES *FGABC1* ACT AS A VIRULENCE FACTOR?

The *F. graminearum* mutant lacking *FgABC1* showed increased sensitivity to the antifungal compounds diamide, salicylanilide and benalaxyl. Diamide is an oxidative agent and can induce oxidative stress in biological systems (Wemmie *et al.*, 1997). Salicylanilide is an uncoupler of oxidative phosphorylation that can inhibit cellular energy production (Williams.Rl & Metcalf, 1967) and benalaxyl is reported to interfere with the synthesis of ribosomal RNA (http://frag.csl.gov.uk/frac_table.cfm). All three of these compounds are active inside the cell and therefore suggest that the *FgABC1* mutant is sensitive to them because of its inability to export them. If the sensitivity of *FgABC1* mutants to the three structurally unrelated compounds is a result of an inability to export them then that would agree with the bioinformatic prediction that this ABC transporter belongs to the subfamily of PDR exporters which are well known to have broad substrate specificities (Sanglard *et al.*, 1996). Unfortunately, the toxic compounds that were identified here as potential substrates for FgABC1 are not known to be produced by plants. Therefore it is still uncertain whether the role of FgABC1 is to counter antimicrobial metabolites produced by the wheat plant. In future it will be important to test possible compounds that have been associated with defences in wheat, these include compounds such as phenolic acids including ferulic acid, coumaric acid and syringic acid as well as benzoxazinoids such as 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA) and 6-methoxy-benzoxazolin-2-one (MBOA) (Moraes *et al.*, 2008).

Wheat plants infected with the *FgABC1* mutant showed significantly less visible FCR symptoms compared to the wild type, but fungal mutant biomass accumulation at 35 dpi was statistically indistinguishable from that of the wild type. Therefore the mutant could still colonise the stem base but could not induce that same degree of plant lesion development. It may be possible that FgABC1 exports compounds that are elicitors or toxins responsible for symptom development, for example, a trigger for programmed cell death in the plant tissue. Fungal toxins can be exported from fungal plant pathogens by ABC transporters, one example being sirodesmin from *Leptosphaeria maculans* (Gardiner *et al.*, 2005). Interestingly, it has also been shown that the PDR transporter of yeast, Pdr5p, is able to export the *Fusarium* toxin deoxynivalenol indicating that PDR transporters like FgABC1 may be able to perform this function (Mitterbauer & Adam, 2002). Another explanation is that the failure of the *FgABC1* mutants to export an exogenously derived wheat compound results in a disequilibrium in disease development processes and reduced symptom and lesion development although this is harder to envisage mechanistically. To study this phenomenon in greater detail it would be interesting to sample the fungal biomass with more replication at more regular intervals throughout the entire FCR infection coupled with histological analysis. This would help clarify if the fungal mutant colonises the host in the same fashion as the wild type in all three phases of infection (Chapter 2).

WHY IS *FGABC1* IMPORTANT FOR FCR BUT NOT FOR FHB?

The overall gene expression profile of *FgABC1* was found to be similar during both FCR and FHB, therefore it was conceivable that the absence of *FgABC1* may have a similar phenotypic effect on *F. graminearum* during both FHB and FCR. This was not the case because it was found that the deletion of *FgABC1* only affected FCR infection. If the fungus was producing a symptom elicitor or toxin then different plant organs may have different levels of response. The pathogenicity tests conducted here were only applied to one wheat cultivar. It was interesting that mutation of the *F. culmorum* gene *FcABC1*, a very close homolog of *FgABC1*, led to much reduced FHB disease development on the wheat variety Remus when compared to wild type but differences were not significant on the varieties Nandu, Hope and Frontana (Skov *et al.*, 2004). This suggests that there may be substantial variation between wheat varieties in their response to *Fusarium* strains with non-functional FgABC1-like genes. Because of this, it is necessary to be cautious in assigning any specificity of function for FgABC1 in FCR and FHB until a broader range of wheat varieties have been screened against the mutants.

FgABC1 has been identified as a virulence factor because it transports one or several compounds that are important for disease development from *F. graminearum* during FCR. Thus, in future it will be important to identify what these compounds are and to functionally characterize them. Once these compounds have been identified, wheat cultivars can be screened in future breeding programs for their degree of tolerance.

It may be argued that *FgABC1* is not a virulence gene in the traditional sense because the fungal mutants lacking the gene could still accumulate wild type amounts of fungal biomass even though they induced significantly less visible symptom. Thus *FgABC1* may be considered a virulence gene functioning as a symptom development factor. Symptom development is a very important part of FCR disease because necrotic lesions at the crown and stem base can cause the wheat heads to dry out prematurely, which can prevent the grain from developing. In a similar light, *Tri5* is considered a virulence gene functioning as a colonisation factor. To date, this is the first *F. graminearum* gene that has shown to be specifically related to FCR of wheat.

Experimental Procedures

VECTOR CONSTRUCTION AND VALIDATION

See Appendix 1, Table A1 for FGSG_04647 and FGSG_01831.

See Table A1 and Figure A1 for FGSG_11164.

See Table A1 and Figure A2 for FGSG_11296.

VECTOR CONSTRUCTION AND VALIDATION FOR *FGABC1*

The *FgABC1* gene replacement vector was created by the PCR split marker method (de Hoogt *et al*., 2000, Fairhead *et al*., 1996). The split marker method involves standard PCR and fusion PCR reactions were to create each half of the knockout cassette as shown in Figure 3.12. Firstly, two flanking fragments from the *FgABC1* locus were amplified including 907 bp of 5' and 811 bp of 3' sequence. The hygromycin resistance cassette was also amplified by the PCR of two overlapping halves with primers as shown in Table 3.4. Hygromycin gene was amplified from vector pHYG1.4Andrew (Mr Andrew Beacham, Rothamsted Research). Cycling conditions used were an initial denaturation step at 95°C for 15 min, followed by 30 cycles of a denaturation step at 94°C for 30 s, an annealing step at 60°C for 30 s and an elongation step at 72°C for 1 min. These cycles were followed by a final elongation step at 72°C for 10min. For all PCR reactions, HotStar Taq® Master Mix was used as per the manufacturer's instructions Figure 3.12.

Table 3.4: Gene-specific primers used to create the 'knock-out' vector for fungal transformation and *FgABC1* gene deletion.

Primer Name	Primer Sequence
ABC F5'	5′-CTAGTGCTTGCAGTTTCTCGAGG-3′
ABC R5'	5′-TCCTGTGTGAAATTGTTATCCGCTATTTCGGGGGAATTTAGGCA-3′
ABC F3'	5′-GTCGTGACTGGGAAAACCCTGGCGTTGAACAAAGGAAAAATGCT-3′
ABC R3'	5′-CACGGAATAGAAATCTTTGG-3′
M13F	5′-CGCCAGGGTTTTCCCAGTCACGAC-3′
M13R	5′-AGCGGATAACAATTTCACACAGGA-3′
NLC37	5′-GGATGCCTCCGCTCGAAGTA-3′
NLC38	5′-CGTTGCAAAACCTGCCTGAA-3′

Next, the PCR products were fused together to create two half-cassettes as shown in Figure 3.12. The 5' flank of *FgABC1* was fused to the first half of the hygromycin gene and the 3' flank was fused to the second half of the hygromycin gene. Cycling conditions used were an initial denaturation step at 95°C for 15 min, then 40 cycles of a denaturation step at 94°C for 30 s, an annealing step at 60°C for 30 s, an elongation step at 72°C for 3 min. The cycles were followed by a final elongation step at 72°C for 10 min.

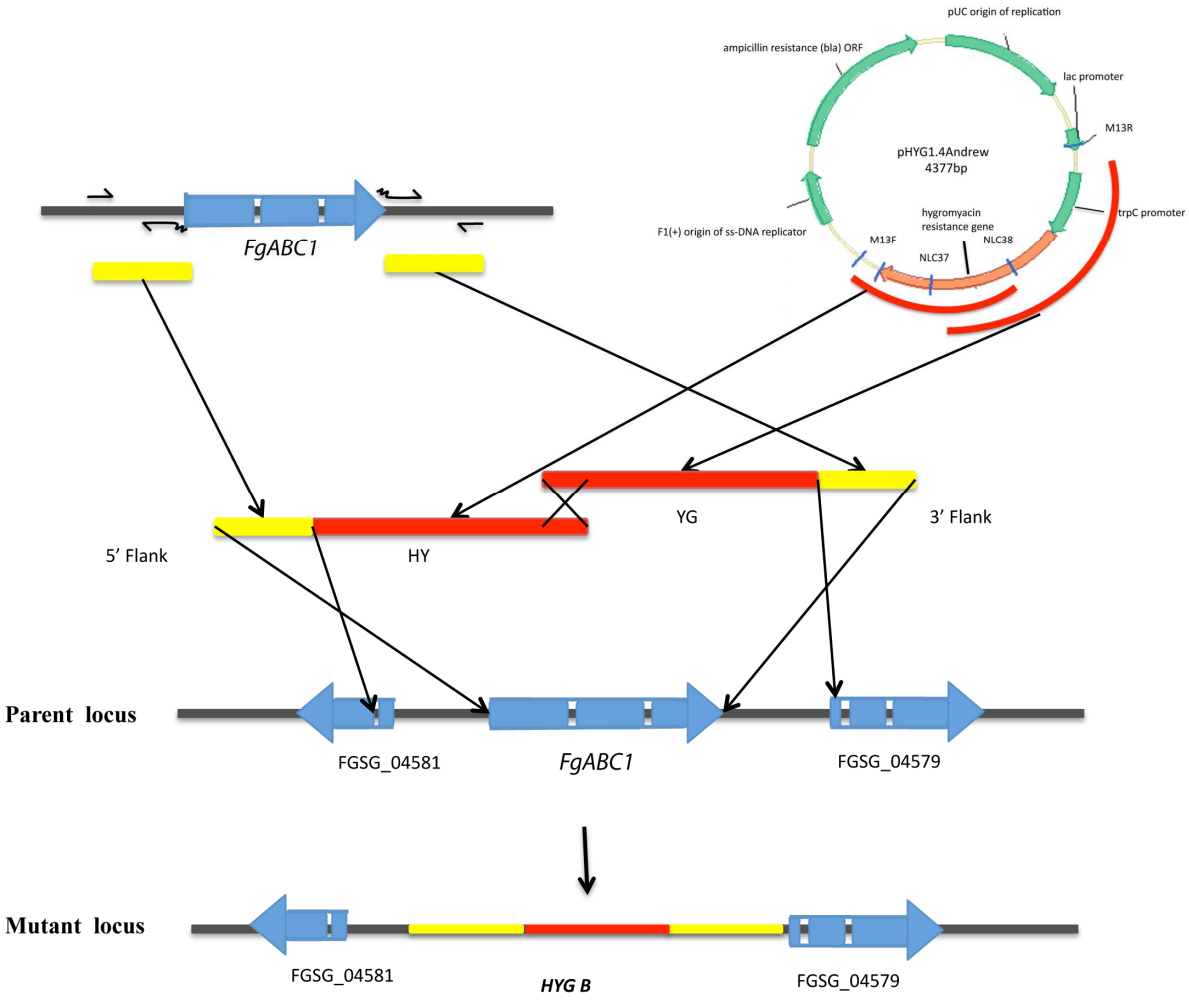

Figure 3.12: Diagram summarizing linear knock-out cassette production. Black arrows represent primer-binding sites.

FUNGAL TRANSFORMATION PROTOCOL

All fungal transformations were completed according to Desmond *et al*, (2008b). Briefly, cell wall degrading enzymes were used to create protoplasts from CS3005 germtubes. These were then infused with the knockout construct DNA, shocked with 1mL of 40% PEG_{4000} and then embedded in regeneration media containing the hygromycin antibiotic. Transformants were screened by PCR amplification of antibiotic resistance gene and native gene sequences. The screen consisted of a multiplex PCR reaction and a southern blot analysis Table 3.5. Due to the split marker system homologous recombination was easily achieved.

Table 3.5: Primers used in the multiplex screen for homologous recombination.

ABCKOscreenF 09	5′ - AACAAAGGCAATGACGGAGT – 3′
ABCKOscreenR 09	5′ - ACCACGTTTAATCCCACCAA – 3′
HygR 09	5′ - CGGTGAGTTCAGGCTTTTTC – 3′

VEGETATIVE GROWTH ASSAY

For vegetative growth assays, a PDA agar plate was inoculated with each fungal isolate (wild type, ΔFgABC1_KO2, ΔFgABC1_KO4 and ΔFgABC1_KO5) and incubated for four days under standard fungal growth conditions. At four days post-inoculation, an agar plug (1cm diameter) was cut out of the outer radius of the fungal colony. A fresh petri dish was inoculated with the plug face down and incubated under standard growth conditions for four days (3 biological replicates per fungal isolate). The diameter of the fungal colony was measured each day to determine the mean growth of the fungus.

FUNGAL STRAIN AND INOCULUM PREPARATION

For the purpose of this report the anamorph term *F. graminearum* will be used to describe the fungus. All experiments described here were conducted with the Australian *F. graminearum* isolate CS3005 (Akinsanmi *et al.*, 2006). Macroconidia for inoculation were produced in the same manner as described in Chapter 2 and spores were used fresh for all inoculations.

PLANT GROWTH

For all experiments the FCR- and FHB-susceptible bread wheat cultivar Kennedy was used. All seedlings were grown in an environmentally controlled glasshouse under the same conditions as described in Chapter 2. When the seedlings were 14 days old they were transferred to an environmentally controlled facility with a constant temperature of 26 ^{o}C and 14 daylight hours for inoculation. For testing crown rot, seedlings were inoculated, scored for disease severity and then harvested at 35 dpi in the same manner as described in Chapter 2.

FCR DISEASE SEVERITY RATING.

Crown rot disease severity of seedlings that were inoculated with the *Fusarium* wild type and mutant strains were calculated using the scoring system outlined in (Mitter et al., 2006b). Briefly, seedlings were grown and inoculated as described above. At 35 dpi the crown rot severity was calculated using the following equation:

Crown rot severity index = (length of stem discoloration**/**seedling height) **x** (number of leaf sheath layers with necrosis)

FHB DISEASE SEVERITY RATING.

Wheat seedlings were drop inoculated with 10 uL of 1x10^{6}/mL *F. graminearum* conidia during anthesis as described by Urban *et al* (2003). There were two independent FHB severity experiments and for both experiments wheat heads were harvested 14 dpi. For the first experiment, FHB disease severity was determined by removing the florets from the rachis and measuring (cm) the length of the necrotic lesion along the rachis, therefore 13 to 15 independent biological replicates per isolate were performed for this experiment. For the second experiment, FHB disease severity was assessed by calculating the percentage of 'bleached' florets for each head as per Urban *et al* (2003) and there were 3 biological replicates per isolate for this experiment.

Chapter 4: *Fusarium graminearum* Cu-Zn superoxide dismutase has a role in virulence during *Fusarium* head blight of wheat

Introduction

Superoxide (O_2^-) is a biologically toxic compound that is formed during the electron transfer processes associated with cellular respiration and photosynthesis as well as during the plant defence response termed the 'oxidative burst'. Superoxide causes damage to cells such as lipid oxidation of membrane structures (Richardson & Korycka-Dahl, 1983) but can be detoxified by the enzyme superoxide dismutase (SOD) (Gort & Imlay, 1998, McCord & Fridovic, 1969). SOD functions by catalyzing the dismutation reaction that converts superoxide into molecular oxygen and hydrogen peroxide (Equation 1). Hydrogen peroxide is less reactive than superoxide radicals but still has some toxicity and generally in cells detoxification is completed by the action of catalase enzymes that convert hydrogen peroxide to water and oxygen.

The 'oxidative burst' of plants occurs as one of the earliest responses to pathogens and pathogen elicitors. The plasmalemma-bound enzyme NADPH oxidase generates superoxide which is then released into the apoplast where its toxicity can potentially inhibit pathogen growth (Zhang *et al.*, 2009). A release of superoxide and other reactive oxygen species (ROS) during an oxidative burst can also serve as a signal that triggers other host defences, such as cross-linking of cell walls (Levine *et al.*, 1994, Bradley *et al.*, 1992). Similarly, hydrogen peroxide produced by SOD has been linked to programmed cell death in plant tissue (Houot *et al.*, 2001). The 'oxidative burst' is also very important in human cells, particularly defensive neutrophils, where ROS are produced in lytic vacuoles to kill sequestered pathogens (Arruda & Barja-Fidalgo, 2009). Therefore the detoxification of superoxide may be generally important for microbial pathogens during the infection process. Consistent with this it has been shown that the superoxide dismutase activity of some pathogenic fungi and bacteria are linked to their virulence and pathogenicity. These pathogens include the human pathogens *Cryptococcus neoformans* (Narasipura *et al.*, 2003) and *Bacillus anthracis* (Cybulski *et al.*, 2009) as well as plant pathogens e.g. *Colletotrichum acutatum* (Brown *et al.*, 2008) and *Botrytis cinerea* (Rolke *et al.*, 2004). Thus far, there is no information available that links the function of SOD to the pathogenicity of *F. graminearum* or any *Fusarium* pathogen.

$$2O_2^- + 2H^+ \xrightarrow{\text{Superoxide dismutase}} H_2O_2 + O_2 \xrightarrow{\text{Catalase}} H_2O + {}^1/_2O_2 \qquad \text{(Equation 1)}$$

There are three main types of SOD present in eukaryotic cells; (I) MnSOD is a manganese-containing enzyme that is localized in the mitochondria (II) Cu-ZnSOD is an enzyme with associated copper and zinc that is localized in the cytosol and (III) another Cu-ZnSOD type that is localized in the extracellular space. All three forms of the SOD enzyme catalyze the breakdown of superoxide anion. In general, fungal species contain only one or two copies of Cu-ZnSOD genes and to date very few have been functionally characterised. There are also some plant pathogens such as *Ustilago maydis* that do not posses any Cu-ZnSOD genes at all. This may indicate that this enzyme may be dispensable for both pathogenicity and development in these fungi. In Chapter 2 a putative Cu-ZnSOD encoding gene of *F. graminearum*, herein termed *FgSOD1*, was identified as being of interest in regards to its expression patterns during infection that suggested a link to virulence or pathogenicity. The expression of *FgSOD1* was higher during the infection of wheat crowns when compared to that observed in axenic culture. Because of its expression in infected plants and its potential function in fungal pathogenicity, the role of *FgSOD1* in FCR and FHB was assessed here using a re-examination of its gene structure and transcription pattern as well as a functional analysis with deletion mutants for this gene.

Results

PHYLOGENETIC ANALYSIS OF *FGSOD1*

It is predicted that there are four superoxide dismutase encoding genes within the *F. graminearum* genome, two MnSOD (FGSG_02051 and FGSG_04454) and two Cu-ZnSOD (FGSG_08721 and FGSG_00576). The two Cu-ZnSOD genes have very different genomic structures and share little homology in the protein sequence. In Chapter 2, one of the Cu-ZnSOD encoding genes (*FgSOD1*, *FGSG_08721*) was found to be up-regulated *in planta* during FCR and was identified as a candidate gene for further functional analysis. This gene was located on *F. graminearum* chromosome 2 and was situated in an area of very low SNP density (Figure 4.1), which suggests that its presence has been evolutionarily stable (Cuomo *et al.*, 2007).

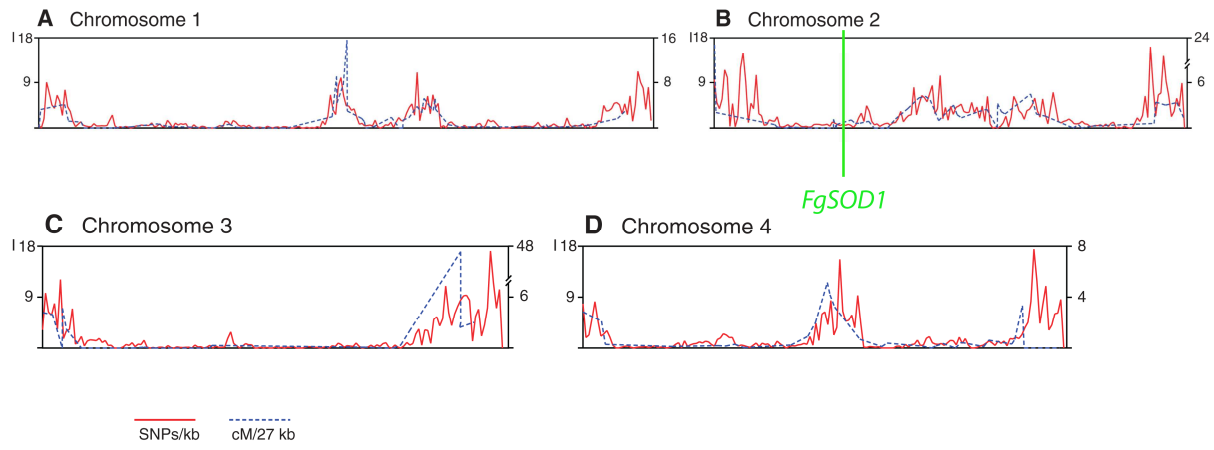

Figure 4.1: Chromosomes 1 to 4 of *F. graminearum* are represented as graphs (A) to (D) respectively. SNP density is shown with a red line and recombination rate is shown as a blue dash. SNPs are referenced to the left y axis as the number of SNPs per kb of high-quality aligned bases and recombination rates are referenced to the right y axis as cM/27kb. Position of *FgSOD1* on chromosome 2 is indicated with a vertical green line. Figure is modified from Cuomo *et al.*, 2007.

The predicted amino acid sequence of FgSOD1 very closely matches that of other Fusaria with 96 % identity to the closest homologues identified in the sequenced genomes of both *F. oxysporum* and *F. verticillioides* (Figure 4.2). However, like *FgSOD1* the function of these *Fusarium* genes has also not been characterised. By protein homology the closest non-*Fusarium* match to FgSOD1 was the CpSOD1 protein of *Claviceps purpurea* that is a known virulence factor (Figure 4.2). The predicted amino acid sequence of FgSOD1 was 96% identical to that of *CpSOD1* that has been shown to have SOD enzymatic activity and to localize extracellularly (Moore *et al.*, 2002). FgSOD1 was also 77% identical at the amino acid sequence level to that of the *Botrytis cinerea* Cu-ZnSOD BcSOD1, which is known to be important for virulence (Rolke *et al*., 2004) (Figure 4.2). When conidia of *B. cinerea* mutant isolates that lacked *BcSOD1* were applied to the primary leaves of young bean plants they developed significantly smaller lesions compared to those produced by the wild type (Rolke *et al*., 2004).

A phylogenetic tree was created to compare the amino acid sequences from diverse fungal Cu-ZnSOD genes (Figure 4.2 B). The analysis included genes from the published genome sequences of non-pathogenic fungi, human pathogenic fungi and plant pathogenic fungi (Figure 4.2). The *Fusarium* species cluster closely with high significance and this parallels the observation that FgSOD1 is evolutionarily conserved. Interestingly the SOD sequence from *Neurospora crassa,* a non-pathogenic fungus co-clusters with a group of pathogenic fungi suggesting that there may not be forms of SOD specialized for pathogenic fungi.

A.

```
F.graminearum_FgSOD1_       MVKAVSVLRGDSKVSGTVVFEQESESAPTTITWDITGNDPNAKRGFHIHT 50
C.purpurea_cpsod1_          MVKAVAVLRGDAKVGGTVVFEQESESAPTTITWDITGNDANAKRGFHIHT 50
B.cinerea_bcsod1_           MVKAVATVRGDSKISGTVTFEQSEENSPTTITWNITGNDANAERGMHVHQ 50
                            *****:.:***:*:.***.***..*.:******:*****.**:**:*:*

F.graminearum_FgSOD1_       FGDNTNGCTSAGPHFNPHNKTHGAPSDETRHVGDLGNVETDGQGNAKGSV 100
C.purpurea_cpsod1_          FGDNTNGCTSAGPHFNPHGKTHGAPTDEARHVGDLGNLETDGQGNAKGSV 100
B.cinerea_bcsod1_           FGDNTNGCTSAGPHFNPHGQTHGAPTDEVRHVGDLGNFKTDAQGNATGSV 100
                            ******************.:*****:**.********.:**.****.***

F.graminearum_FgSOD1_       TDSLIKLIGPHSVIGRTVVIHAGTDDLGKGDGEESLKTGNAGPRPACGVI 150
C.purpurea_cpsod1_          KDEHVKLIGPHSVIGRTVVIHAGTDDLGKGDNEESLKTGNAGPRPACGVI 150
B.cinerea_bcsod1_           QDSHIKLIGPLSVIGRTVVVHSGTDDLGKGENEESKKTGNAGTRPACGVI 150
                             *. :***** ********:*:********:.*** ******.*******

F.graminearum_FgSOD1_       GISN 154
C.purpurea_cpsod1_          GISS 154
B.cinerea_bcsod1_           GIAA 154
                            **:
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B.

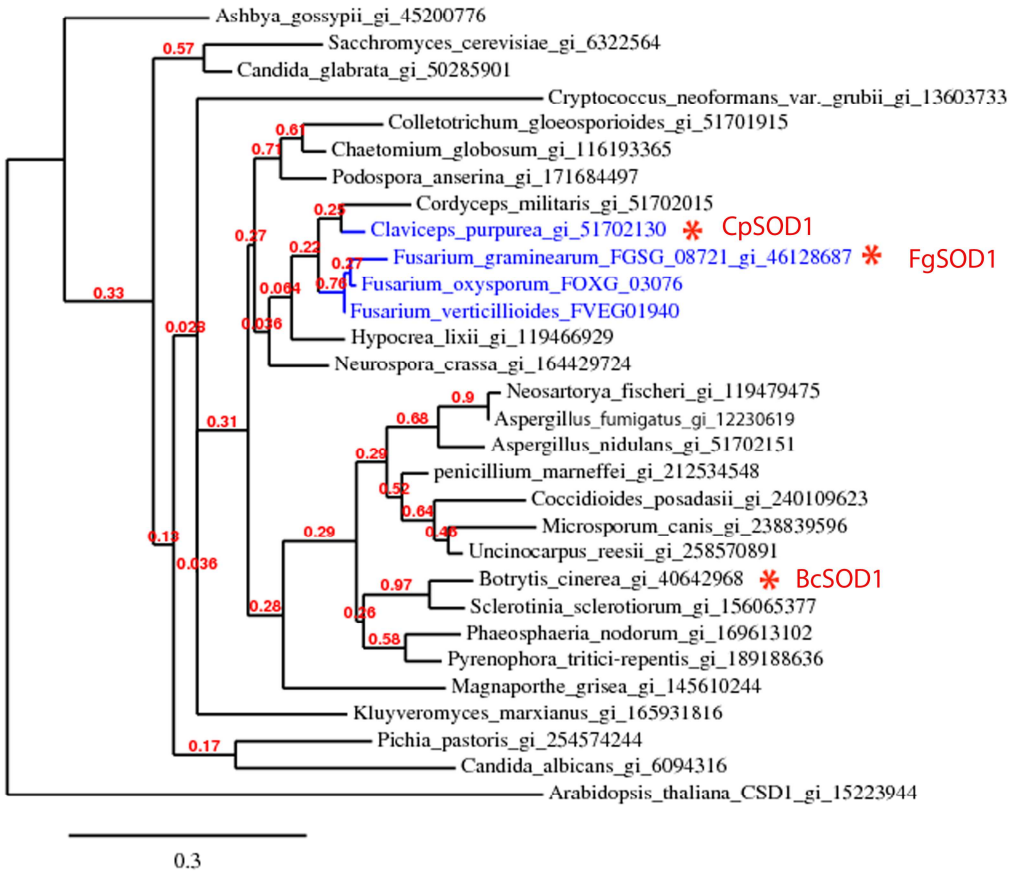


Figure 4.2: Sequence analysis of FgSOD1. (A) ClustalW2 amino acid sequence alignment between FgSOD1, BcSOD1 (GenBank Accession: CAD88591) and CpSOD1 (GenBank Accession: CAC50073). FgSOD1 is 77% and 96% identical to BcSOD1 and CpSOD1, respectively. (B) Phylogeny tree of predicted Cu-ZnSOD protein sequence. Clustering of *C. purpurea* and *Fusarium* species is shown in blue. The SOD from the alignment in A. above are marked with a red * in B. and a plant-derived SOD sequence from *Arabidopsis thaliana* was included as an outgroup.

ANALYSIS OF *FGSOD1* TRANSCRIPT LEVELS AND PROCESSING

Analysis of *FgSOD1* transcripts during both FCR and FHB indicated that there were two distinct expression profiles depending on the location within the gene of the probe set used to measure expression (Figure 4.3). In fact, these expression profiles seemed to be 'mirror-images' of each other. This type of 'mirror image' expression profile has shown to be strongly associated with alternative splicing in other studies (Le *et al.*, 2004, Kawai *et al.*, 2005). While alternative splicing has been reported as rare, it has been detected in at least one other fungal plant pathogen, the rice blast pathogen *M. grisea* (Ebbole *et al.*, 2004). The expression patterns shown in Figure 4.3 suggest that there may be alternative splicing of *FgSOD1* and the dominant alternative splice form changes during infection as explained below.

The *F. graminearum* Affymetrix GeneChip array contains three different probe sets for *FgSOD1* located at different sites of the gene (Figure 4.4). Probe set 1 (fgd353-190_at) was located at the 5' end of the first intronic region according to the current annotation (FG3), probe set 2 (fg08721_at) was located at the 3' end of the first intronic region and probe set 3 (fg08721_s_at) was located across exon numbers 2, 3 and 4. Results from the analysis of global gene expression during FCR (Chapter 2) and during FHB of barley (Guldener *et al.*, 2006b) show that *FgSOD1* had generally similar transcription profiles during both FCR of wheat and FHB of barley (Figure 4.3). For example, probe set 1 was transcribed to a higher degree early in infection compared to axenically cultured mycelia and decreased significantly over the course of the wheat infection. This suggests that an alternate transcript of the gene may have been dominant at early stages of infection. Probe set 2 produced very low signal across all time points of FCR and FHB infection suggesting that this sequence was not expressed at any time during the infections. However, probe set 3 was transcribed lower early in infection compared to axenically cultured mycelia and increased significantly over the course of infection. This would therefore imply that transcripts encoding the predicted FgSOD1 protein (i.e. fully spliced transcripts) may have been increasing during infection. The only major difference between the expression patterns in FCR and FHB was that the scale of change in expression during FCR was much greater than during FHB and therefore results for FCR were plotted using a logarithmic scale in Figure 4.3. As an additional control for the specificity of the expression data obtained using arrays, the individual probe sequences for all probe sets were analysed by BLAST to identify if there could be any cross hybridization to another part of the fungal genome. This confirmed that all probe sequences were unique to the *FgSOD1* gene.

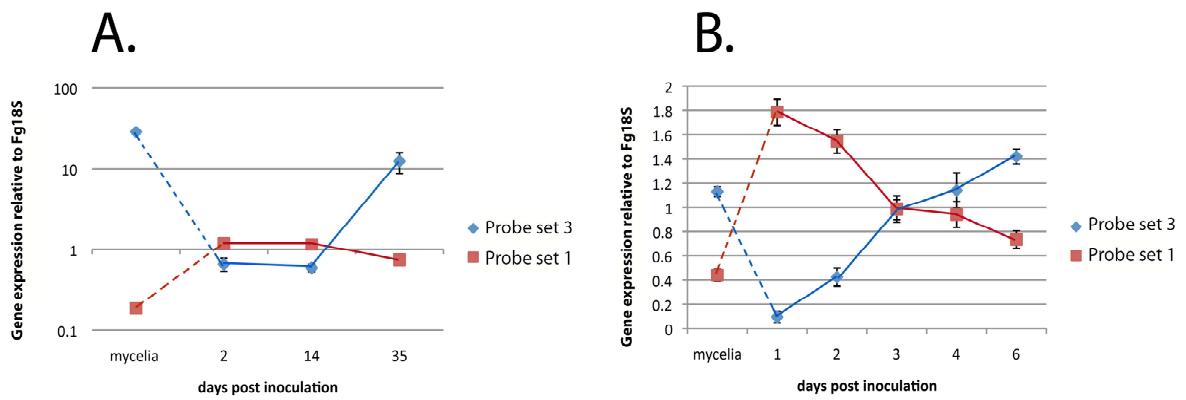


Figure 4.3: *FgSOD1* expression as assessed by Affymetrix GeneChip experiments (A) during FCR of wheat Chapter2: (Stephens *et al.*, 2008) (B) during FHB of barley (Guldener *et al*., 2006b). Error bars are the standard error of the mean for three to four biological replicates. Error bars for mycelial culture data-points are too small to be visible. Scale for *FgSOD1* expression during FCR is logarithmic.

GENERATION AND VALIDATION OF *FGSOD1* DELETION MUTANTS

In order to test if *FgSOD1* plays a role in pathogenicity or virulence during FCR or FHB, whole gene deletion mutants were created using the split-marker method as per Chapter 3 (de Hoogt *et al*., 2000, Fairhead *et al*., 1996). The open reading frame of the whole gene was deleted in order to account for any alternative forms of transcription *FgSOD1* may have and this is discussed later in this chapter. The protoplast transformation protocol yielded 15 independent colonies and when screened by PCR all 15 showed homologous recombination resulting in the replacement of the whole *FgSOD1* gene with the antibiotic resistance cassette. Three of these isolates were chosen FgSOD1_KO1, FgSOD1_KO2 and FgSOD1_KO3 to use in further phenotype tests (Figure 4.5). All three *FgSOD1* mutants were tested visually for conidia, perithecia and ascospore formation and there were no defects observed during these reproductive stages of the fungal life cycle.

Figure 4.4: Genomic nucleotide sequence of *FgSOD1*. Start and stop codons are highlighted in red. Exons in the current FG3 annotation are highlighted in yellow. Colored lines below the genomic DNA sequence indicate probe sets; probe set 1 (fgd353-190_at) are represented as red lines, probe set 2 (fg08721_at) are green lines and probe set 3 (fg08721_s_at) as dark blue lines.

To test whether the mutant isolates incurred any vegetative growth defects directly due to the transformation process, growth rates of the mutants were compared to the wild type on complete and minimal media. Results show that there was no significant difference in growth rates between the wild type and all three mutant strains on a defined minimal and complete (V8 juice) nutrient agar medium (Figure 4.6).

Paraquat (N,N'-dimethyl-4,4'-bipyridinium dichloride) induces endogenous superoxide production in many biological systems including mammalian (Gage, 1968), plants (Dodge, 1971), bacteria (Hassan & Fridovich, 1978) and fungi (Smith & Lyon, 1976). Within the cell there is a cyclic reduction of paraquat that facilitates the production of activated oxygen such as the superoxide anion (Bus & Gibson, 1984). In order to test the sensitivity of the *FgSOD1* mutants to internal oxidative stress their rate of growth was measured in the presence of enriched ROS generated by exposure to paraquat and compared to that of the wild type. To do this, agar plates were supplemented with a range of concentrations of paraquat including 0mM, 1mM and 10mM and the radial growth rates of the *F. graminearum* isolates were measured daily. The *FgSOD1* mutant strains were only slightly inhibited in their ability to grow on agar supplemented with paraquat, and their growth was variable. The growth difference from controls was and not statistically significant (Figure 4.7). This indicates that the mutants were not different from wild type in their sensitivity to internally generated superoxide. One explanation for this result is that the location of the majority of the *FgSOD1*-encoded superoxide dismutase may not be intracellular. This would be consistent with the strong amino acid sequence homology (Figure 4.2) of FgSOD1 to CpSOD1, which is known to be primarily extracellular (Moore *et al*., 2002).

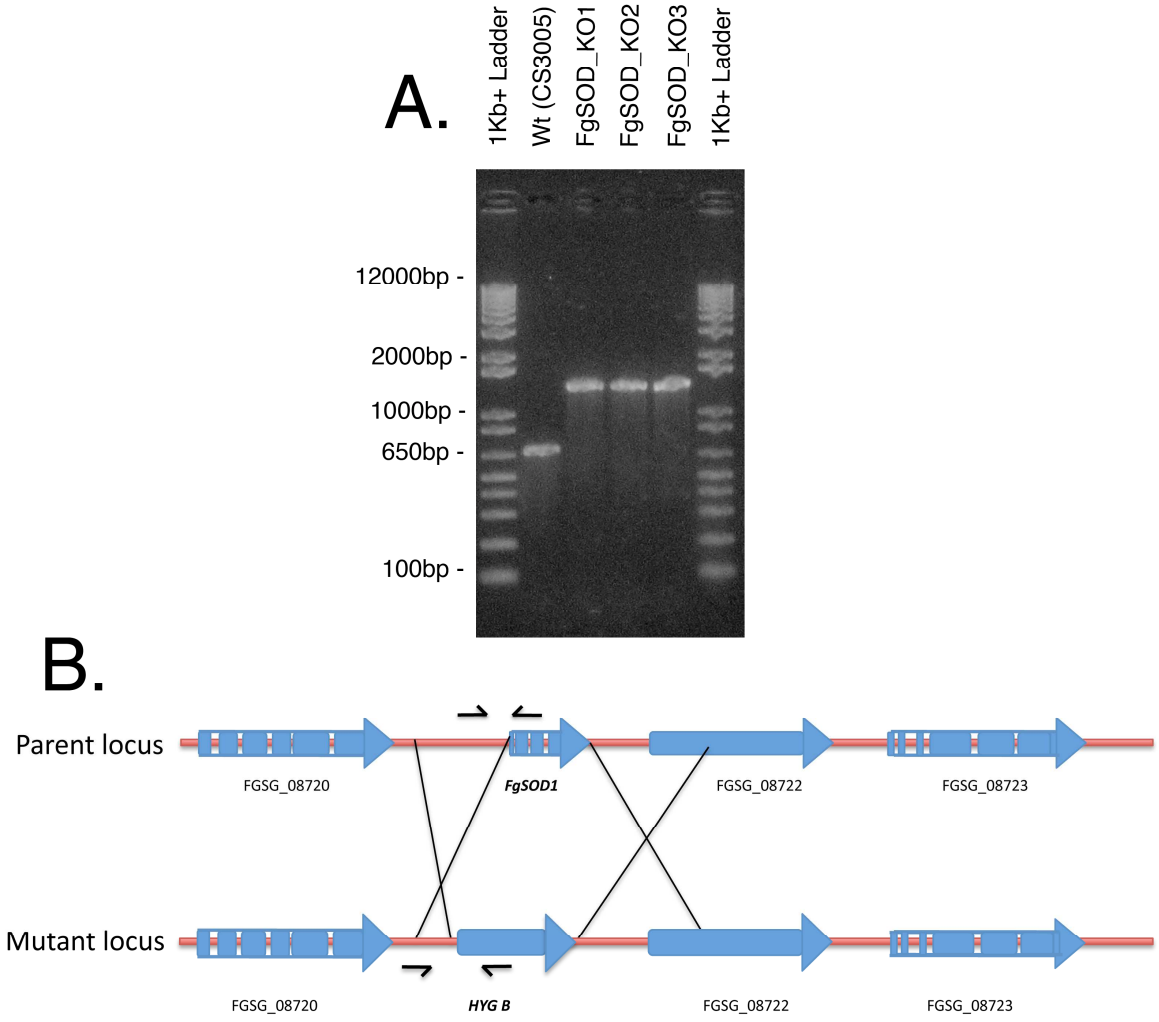


Figure 4.5: *FgSOD1* mutant validation by PCR screen. (A) amplification of *F. graminearum* wild type and *FgSOD1* mutant genomic DNA using multiplex PCR to show homologous recombination has resulted in the deletion of the complete *FgSOD1* open reading frame. (B) represents the transformation event and location of the multiplex PCR screening primers. In the PCR screen the wild type amplifies as a 659 bp fragment and mutant amplifies as a 1345 bp fragment. An ectopic recombination would result in the amplification of both fragments.

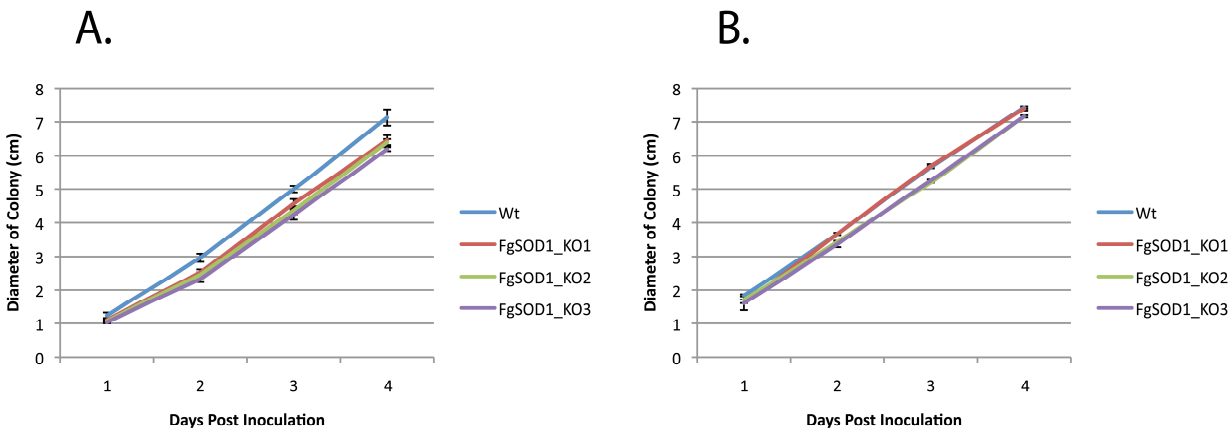


Figure 4.6: Vegetative growth of *F. graminearum* Wt (CS3005) and *FgSOD1* mutants on (A) defined low nutrient media (SNA) and (B) complex media (V8 juice agar). All error bars are the standard error of the mean for three independent biological replicates.

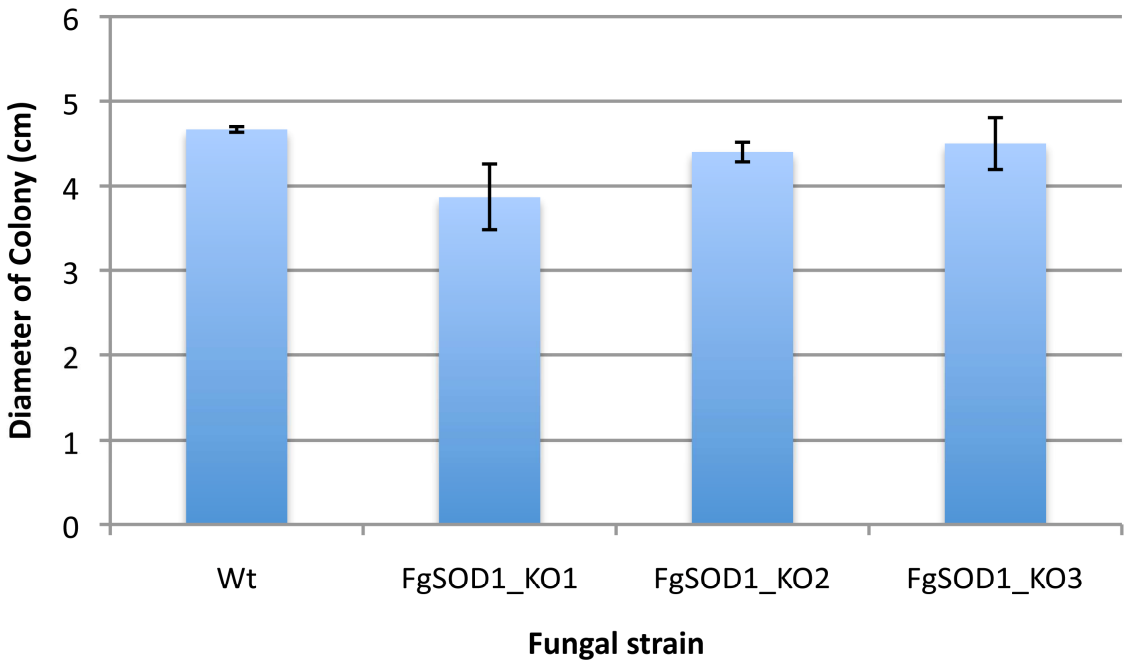


Figure 4.7: Growth of Wt and FgSOD1 mutant isolates at 4 dpi in the presence of internal superoxide stress induced by paraquat (10mM). Error bars are the standard error of the mean for three independent replicates.

FgSOD1 IS DISPENSABLE FOR FCR DISEASE OF WHEAT

After confirming the 3 independent *FgSOD1* mutants did not have any *in vitro* growth or sporulation defects, the isolates were then tested in a crown rot bioassay to assess the importance of *FgSOD1* for *F. graminearum* virulence or pathogenicity during FCR. To do this, wheat seedlings were drop inoculated with conidia and crown rot disease severity was assessed at 35 dpi (Mitter *et al*., 2006b). For each isolate 25 to 30 biological replicate plants were tested. Comparison of the calculated crown rot severity indices showed that there was no significant difference in visible crown rot symptoms between the wild type and any of the three *FgSOD1* mutants (Figure 4.8). All three mutants showed a trend towards an increased crown rot severity index, although this was not statistically significant. This indicates that Cu-Zn superoxide dismutase is not essential for the development of the typical necrotic lesions which are symptoms of a FCR infection in wheat.

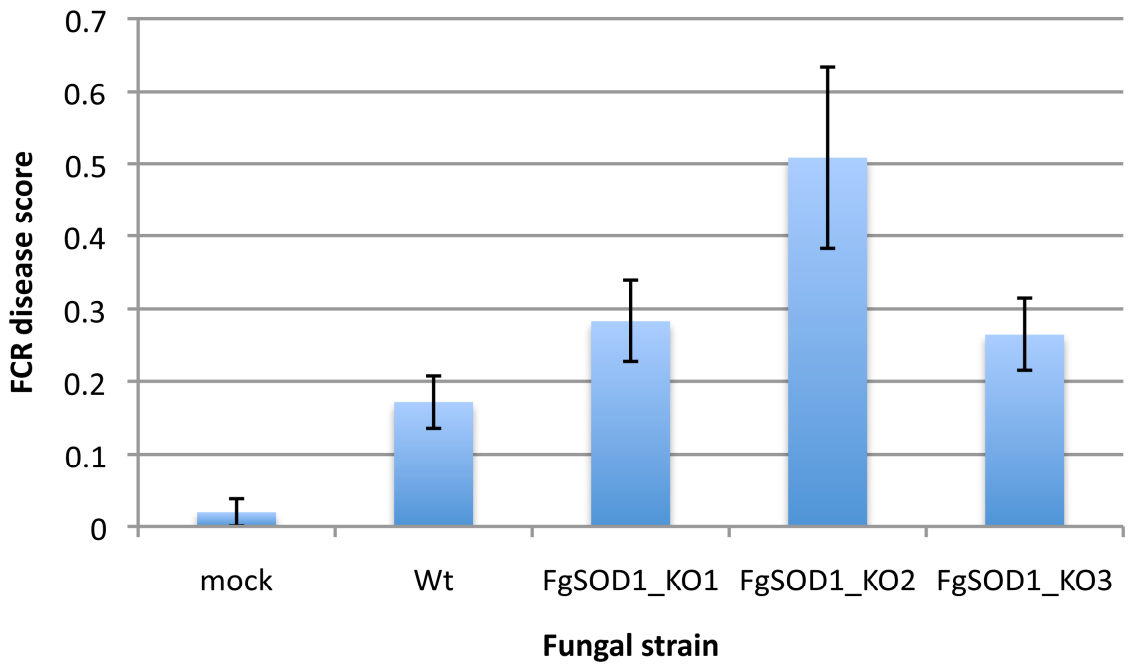
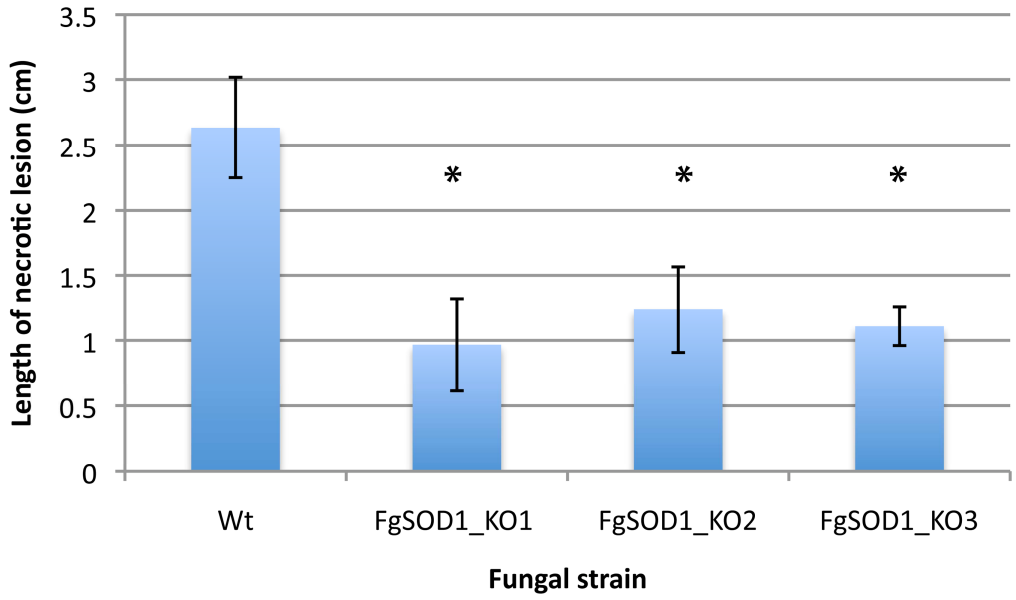


Figure 4.8: Visual crown rot severity score for FCR of wheat infected with water (mock), wild type (CS3005) *F. graminearum* and the three independent *FgSOD1* mutants. All error bars are the standard error of the mean for 25 to 30 independent biological replicates.

FgSOD1 IS AN IMPORTANT VIRULENCE FACTOR DURING FHB OF WHEAT

Although *FgSOD1* was not essential for in FCR, its importance in FHB disease was also tested. To do this, wheat heads were point inoculated at mid-anthesis and disease severity was assessed 14 dpi by measuring the length of the necrotic lesion on the rachis. The three independent mutants and the wild type were used with 14 to 16 biological replicate plants for each isolate. Interestingly, wheat heads inoculated with *FgSOD1* mutants developed significantly less visible symptoms compared to the wild type inoculated heads (Figure 4.9). This suggests that *FgSOD1* may play an important role in the virulence of *F. graminearum* during FHB of wheat. These results were confirmed by a smaller independent experiment with only three biological replicates per isolate. In this experiment wheat heads were point inoculated in the same manner and the degree of bleaching was assessed. On average, FHB caused by the wild type resulted in 98.2% bleaching of the wheat heads whereas FgSOD1_KO1 resulted in 39.5%, FgSOD1_KO2 = 28.7% and FgSOD1_KO3 = 78.2%.

A.



B.

Figure 4.9: Assay of *F. graminearum* pathogenicity during FHB disease in wheat. All error bars are the standard error of the mean for 13 to 15 biological replicates. *T-tests* showed statistically significant changes in the development of FHB between the wild type and the three mutants. * indicates *P value* = 0.003 between the Wt (CS3005) and FgSOD1_KO1, 0.01 between the Wt and FgSOD1_KO2 and 0.001 between the Wt and FgSOD1_KO3 (A). Representative images of FHB pathogenicity assays of *F. graminearum* isolates (B). (i) Wt (CS3005) (ii) FgSOD1_KO1 (iii) FgSOD1_KO2 (iv) FgSOD1_KO3.

Discussion

Reactive oxygen species, including superoxide, are part of a classical plant defence response in host-pathogen interactions (Doke, 1983). Importantly, the results presented in this chapter demonstrate that *FgSOD1* plays an important role in virulence during FHB of wheat (Figure 4.9). However, a role for *FgSOD1* was not observed in FCR where disease the virulence from mutants lacking this gene was not statistically different from that of the wild type strain (Figure 4.8). In this study it was shown that *FgSOD1* was important for the virulence of *F. graminearum* during FHB of wheat, but not during FCR suggesting that ROS may play a differential role in the host-pathogen interactions for FCR and FHB.

HOW IS FGSOD1 ACTING AS A VIRULENCE FACTOR DURING FHB?

In vitro growth experiments demonstrated that the reduction in virulence observed in *FgSOD1* mutants was not due to the inhibition of vegetative growth or asexual/sexual reproduction. This indicates that the observed phenotype of reduced virulence in FHB can be attributed specifically to a defect in the infection process and not to a general inability to grow. *FgSOD1* could be acting as a virulence factor by detoxifying superoxide that would otherwise be toxic to the fungal cell and inhibit growth. Both host and pathogen are possible sources of superoxide during the host-pathogen interaction. If the superoxide were endogenous then the SOD enzyme would be located in the fungal cell, whereas if it were host-derived the SOD may function in the extracellular space. While it is not known where the enzyme activity of this SOD is located, bioinfomatic analysis of its closest homologue in other fungal species indicates that it may be in the extracellular space.

When the *FgSOD1* mutant strains were challenged with internal oxidative stress induced by paraquat their vegetative growth was not significantly inhibited compared to the wild type. Paraquat induces oxidative stress by undergoing cyclic reduction and generating superoxide within the cell by. Thus, if the FgSOD1 superoxide dismutase were working only in the extracellular space then loss of this enzyme activity would not significantly affect the ability of the fungus to detoxify the superoxide induced by paraquat. Loss of the highly homologous extracellular CpSOD1 in *C. purpurea* also resulted in no change in tolerance to internal superoxide generated using

paraquat (Moore *et al*., 2002). In future functional studies beyond the scope of this PhD is will be important of identify where FgSOD1 is localised. In order to reliably achieve this a study may be required as was done by (Moore et al., 2002) where the fungus was grown in axenic culture and the fractions were separated and immunoassayed with polyclonal antibodies raised against a Cu-Zn SOD from *C. fusiformis*.

Fungal pathogens can produce extracellular Cu-Zn superoxide dismutase enzymes, one of which includes *Candida albicans* (Frohner *et al.*, 2009). These extracellular Cu-Zn superoxide dismutase enzymes possess signal peptides and are Glycophosphatidylinositol (GPI)-anchored at the C-terminus. However, the current annotation of *FgSOD1* and its proposed product does not indicate the presence of a signal peptide. Interestingly, there have been a number of reports of some fungi producing extracellular superoxide dismutase with no obvious signal peptide (Rolke *et al*., 2004, Moore *et al*., 2002). Alternative modes of transport for the SOD enzyme to outside of the cell have been suggested which include; alternative splicing of the mRNA transcripts in such a way as to introduce a transmembrane segment into the polypeptide (Fujii *et al.*, 1998), or utilisation of an alternative transport system that does not require any of the known signal peptides.

The current annotation of *FgSOD1* is slightly ambiguous with multiple Affymetrix probe sets exhibiting transcription profiles that are mirror images of each other. Assuming the phenomena described is indeed alternative splicing then it seems that *FgSOD1* could encode two forms of superoxide dismutase. One form (indicated by probe set 1) is expressed in the early stages of infection when the plant-derived oxidative burst occurs and the other form (indicated by probe set 3) is expressed at later stages of infection. There are many *F. graminearum* EST sequences for this gene that concur with the coding sequence provided in the current annotation and probe set 3, but all of these EST clones were obtained from axenically cultured mycelia or mycelia harvested from plants very late in infection (www.broadinstitute.org). Both of these experimental conditions may favour the transcription pattern detected by probe set 3. At this stage there is not enough information to propose the structure of any alternate forms for *FgSOD1*. More work is needed to address the structure of both possible mRNA transcripts and proteins encoded from this gene, as it appears to be important for *F. graminearum* virulence. This could be achieved by creating a fungal cDNA library during early and late stages of infection or by proteomic analyses.

Fusarium may use superoxide dismutase to protect itself against plant-derived ROS generated by the oxidative burst. Superoxide dismutase detoxifies superoxide, but in the process it also produces

hydrogen peroxide (H_2O_2). Hydrogen peroxide has been observed in wheat leaves inoculated with *F. pseudograminearum* (Desmond *et al*., 2008b), which suggests either the host or pathogen may produce ROS during the interaction. However, the production of hydrogen peroxide by DON treatment (in the absence of pathogen) indicated that at least some of the hydrogen peroxide is of plant origin during *Fusarium* infections. For host-produced ROS, the role of *FgSOD1* may be to either (i) contribute to the reduction of superoxide that is toxic to the fungus or (ii) to generate hydrogen peroxide that may have a signalling role in plant defence. Plant pathogenic fungi have been observed to produce reactive oxygen species during infection (Egan *et al.*, 2007). Indeed Desmond *et al,*. (2008b) observed staining for hydrogen peroxide in the apical cells of *F. pseudograminearum* macroconidia when inoculated onto plants. FgSOD1 may play a role in producing hydrogen peroxide that acts as a signal for the host to undergo programmed cell death thereby making nutrients available for the necrotrophic lifestyle of the fungal pathogen.

Taken together, the above possibilities suggest that FgSOD1 may have a role in virulence for FHB by either detoxifying host-produced superoxide that is toxic to the fungus or by generating hydrogen peroxide using superoxide of plant or fungal origin in order to promote cell death in the host.

WHY DOES FGSOD1 ASSIST VIRULENCE DURING FHB BUT NOT FCR?

Superoxide dismutase may be acting as a virulence factor by detoxifying superoxide. If this is the case then it can be reasonably argued that *FgSOD1* plays a more significant role in virulence during FHB compared to FCR due to more superoxide being produced in the wheat florets compared to that produced in the crown and stem base tissue. Evidence of significant oxidative burst has been described in the heads of wheat infected with *F. graminearum* (Zhou *et al.*, 2005). There is also evidence of a ROS response being induced in wheat leaf tissue upon application of the closely related fungal species *F. pseudograminearum* (Desmond *et al*., 2008b). However, there have not been any published reports comparing the degree of superoxide generation between wheat crown and head tissue during *Fusarium* infection. Global gene expression analysis of wheat head tissue showed that reproductive organs exhibit a significantly different set of transcription profiles compared to vegetative organs (Golkari *et al.*, 2007). Therefore, it is conceivable that host defence responses such as ROS production have evolved to be induced more highly in the wheat head compared to the crown tissue. Clearly, a study of the levels and composition of ROS species

produced in the head and crown during *Fusarium* infection development is needed to resolve this question. There was inadequate time available in this PhD study to undertake this analysis.

From the studies described in Chapters 3 and 4 it has been shown that the *F. graminearum* genes *FgABC1* and *FgSOD1* are important for fungal virulence in wheat. It is interesting that these two genes each seem to show specificity for one of the two *Fusarium* diseases of the wheat plant. That is, *FgABC1* was specifically linked to FCR and *FgSOD1* was specifically linked to FHB. However, what this may indicate is that there is a set program of transcription throughout the infection for virulence-associated genes and this set program is similar for all *Fusarium* infections. Despite a common transcriptional regulation these gene products are not necessarily essential for both modes of infection. Genes that are essential for all *Fusarium* infections may make up core group of virulence genes and other genes may be specialized for a particular infection. This idea of a core group and an outer group of virulence and pathogenicity genes can be visually represented as a Venn diagram as shown in Figure 4.10. In this diagram *FgABC1* is an FCR-specific virulence gene and therefore sits on the left hand side and *FgSOD1* is an FHB-specific virulence gene and sits on the right. Further analysis of specific genes is needed to populate this diagram more comprehensively.

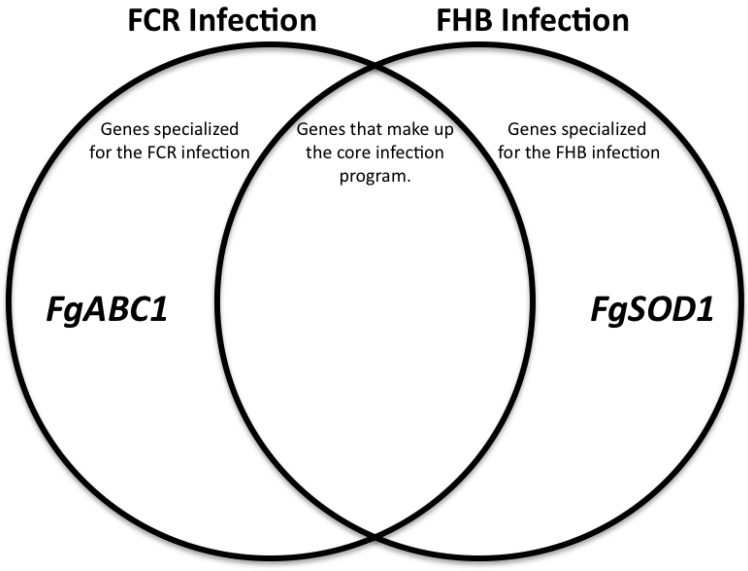


Figure 4.10: Venn diagram showing the three different categories that *F. graminearum* virulence and pathogenicity genes can fall into. Here *FgSOD1* is classified as a gene that is specialized for function during FHB infection and *FgABC1* is classified as a gene that is specialized for FCR.

Experimental Procedures

VECTOR CONSTRUCTION AND VALIDATION

Creation of knock-out vectors was performed using the same split marker protocol as described in Chapter 3 using the *FgSOD1*-specific primers listed in Table 4.1.

Table 4.1: Gene specific primers used to create the 'knock-out' vector for fungal transformation and *FgSOD1* gene deletion.

Primer Name	Primer Sequence
FgSOD1 F5'	5′-CTAGTGCTTGCAGTTTCTCGAGG-3′
FgSOD1 R5'	5′-TCCTGTGTGAAATTGTTATCCGCTATTTCGGGGGAATTTAGGCA-3′
FgSOD1 F3'	5′-GTCGTGACTGGGAAAACCCTGGCGTTGAACAAAGGAAAAATGCT-3′
FgSOD1 R3'	5′-CACGGAATAGAAATCTTTGG-3′

FUNGAL TRANSFORMATION PROTOCOL

Fungal transformations were completed according to (Desmond *et al.*, 2008b). Transformants were screened by PCR of antibiotic resistance gene and native gene as described in Chapter 3 using the *FgSOD1* specific primers in Table 4.2.

Table 4.2: Primers used in the multiplex screen for homologous recombination.

SODKOscreenF 09	5′- AGCGAGTTTCTGAGCTCTCG -3′
SODKOscreenR 09	5′- CCGGAGAATTATGCAGGAAC -3′
HygR 09	5′- CGGTGAGTTCAGGCTTTTTC -3′

VEGETATIVE GROWTH AND GROWTH ON PARAQUAT ASSAYS.

For vegetative growth assays, a PDA agar plate was inoculated with each fungal isolate (wild type, FgSOD1_KO1, FgSOD1_KO2 and FgSOD1_KO3) and incubated for four days under standard fungal growth conditions 22^{o}C in 12/12h day-night cycle of lighting. At four dpi an agar plug was cut out of the radius of the fungal colony (1 cm diameter), a fresh 9 cm PDA petri dish (inoculated with 10mM paraquat for oxidative stress test) was inoculated with the plug face down and incubated under standard growth conditions for four days (3 replicates per fungal isolate). The diameter of the fungal colony was measured each day.

FUNGAL STRAIN AND INOCULUM PREPARATION

All experiments described here were conducted with the Australian *F. graminearum* isolate CS3005 (Akinsanmi *et al*., 2006) as per Chapter 3.

PLANT GROWTH, INOCULATION AND HARVESTING TECHNIQUE

For all experiments the FCR- and FHB-susceptible bread wheat cultivar Kennedy was used. All seedlings were grown, inoculated and harvested in the same way as described in Chapter 3.

FCR DISEASE RATING

Wheat seedlings were inoculated and rated for FCR using the same method as described in Chapter 3. For each *F. graminearum* isolate 30 seedlings were inoculated for independent biological replicates.

FHB DISEASE RATING

Wheat seedlings were inoculated with *F. graminearum* macroconidia and harvested during anthesis into a middle floret and FHB disease was scored in the same manner as described in Chapter 3.

CHAPTER 5: Testing a role in FCR for known pathogenicity and virulence genes involved in FHB

Introduction

It was highlighted in Chapters 3 and 4 that *FgABC1* and *FgSOD1* show specificity for one of the two diseases. This is interesting not only from a biological point of view but also from a practical point of view in that a method of controlling FHB using genetic control aimed at *FgSOD1* would probably not be useful for FCR and vice versa with *FgABC1*. Previously, FHB has been extensively researched as a stand-alone disease and a suite of genes have been discovered that are involved in the virulence and pathogenicity of *F. graminearum* during FHB (www.phi-base.org). However, the possible role of these genes in FCR is unknown. This study was therefore designed to test if genes that are known virulence factors during FHB are also virulence factors during FCR. Similar gene requirements for the fungus during FHB and FCR disease development would support the observations from gene expression analysis shown in Chapter 2 that indicate possible similarities at early stages of infection and suggest possible common modes of function for infection. Because all previously published deletion mutants of *F. graminearum* have been developed outside of Australia and quarantine regulations forbid the infection of plants with exotic strains, these experiments were conducted in the U.K. at Rothamsted Research in collaboration with the laboratory of Dr. Kim Hammond-Kosack. At Rothamsted Research a collection of fungal mutants is held that have impaired virulence on wheat for FHB as described below. Therefore, FCR bioassays were performed with these gene deletion mutant strains for each gene in conjunction with the corresponding wild type.

The FHB-related genes tested:

(*Map1*) FGSG_06385: This gene encodes a mitogen-activated protein kinase that is a signalling molecule and homologs have been shown to be essential for penetration and primary infection in many pathogenic fungi. (Urban *et al*., 2003, Jenczmionka *et al*., 2003)

(*Tri5*) FGSG_03537: Trichodiene synthase. *Tri5* facilitates the first step in the biosynthesis of the DON toxin (Proctor *et al*., 1995). *F. graminearum* isolates that lack this gene show reduced virulence during FHB.

(*Top1*): Is a probable subunit of an anaphase-promoting complex and is part of the cyclosome. Unpublished work by Dr Thomas Baldwin at Rothamsted Research therefore gene accession number is not provided.

(*Nth1*): probable neutral trehalase. Trehalase is an enzyme that is involved in the metabolism of the sugar molecule trehalose. This mutant came from unpublished work by Andrew Beacham at Rothamsted Research therefore gene accession number is not provided.

(*Snf1*): Is a probable serine/threonine protein kinase. It is an ortholog of the SNF1 protein kinase in yeast, which is an important gene in glucose-repressible gene expression during glucose starvation. In *F. graminearum* it has been shown to be necessary for the sexual cycle and spore development (Lee *et al.*, 2009). This mutant came from unpublished work by Dr Martin Urban at Rothamsted Research therefore gene accession number is not provided.

(*Fgl1*) FGSG_05906: Is a gene that probably encodes a precursor to a triacylglycerol lipase enzyme. Lipases are enzymes that metabolize and break down fat molecules and this gene is involved in extracellular lipolytic activity (Voigt *et al*., 2005). *F. graminearum* mutants that lack this gene show reduced virulence during FHB.

(*Hog1*) FGSG_09612: Is a probable osmotic sensitive-2 protein (putative mitogen-activated protein (MAP) kinase homolog). This gene is involved in responding to hyperosmotic stress (Ramamoorthy *et al.*, 2007).

Comparing the disease severity of FCR and FHB caused by infection with these mutants would give some insight into whether similar genes and processes are required for these two diseases or whether distinct mechanisms are operating.

Results

To investigate if FHB virulence genes of *F. graminearum* also play a role in virulence during crown rot, wheat seedlings were inoculated at the stem base with wild type and mutant isolates to induce a crown rot infection. Crown rot disease severity was assessed on the bases of visual symptoms at 28 days post inoculation (dpi) according to the crown rot severity index (Mitter *et al*., 2006b). This work was completed at Rothamsted Research, England using the FHB susceptible wheat cultivar Bob White. Because this host cultivar and the non-Australian wild type strains of *Fusarium* had not been used in FCR infection experiments before it was important to first demonstrate that compatible interactions could be established.

FCR SYMPTOM DEVELOPMENT CAUSED BY DIVERSE *FUSARIUM* WILD TYPE STRAINS

Five different wild type *Fusarium* species were analysed in this experiment including;

PH-1: The USA *F. graminearum* isolate that was used for the genome sequence analysis (Cuomo *et al*., 2007).

1003: An English *F. graminearum* isolate

16A: A German *F. graminearum* isolate

98-11: An English *F. culmorum* isolate

CS3096: An Australian *F. pseudograminearum* isolate from crown rot infected wheat that had been previously sent to the UK and was used as a positive control.

Compared to the other wild type isolates, *F. pseudograminearum* produced the most severe visual symptoms with an average FCR severity score of 0.543 (Figure 5.1). However, the *F. graminearum* isolates PH-1, 16A and the *F. culmorum* isolate all exhibited the ability to elicit visual symptoms and significant scores for crown rot disease with ratings of 0.21, 0.12 and 0.16, respectively. Finally, the English *F. graminearum* isolate 1003 produced the least severe crown rot symptoms with a rating of 0.031. These results indicate that the wild type strains were all capable of causing FCR disease symptoms but of varying intensity.

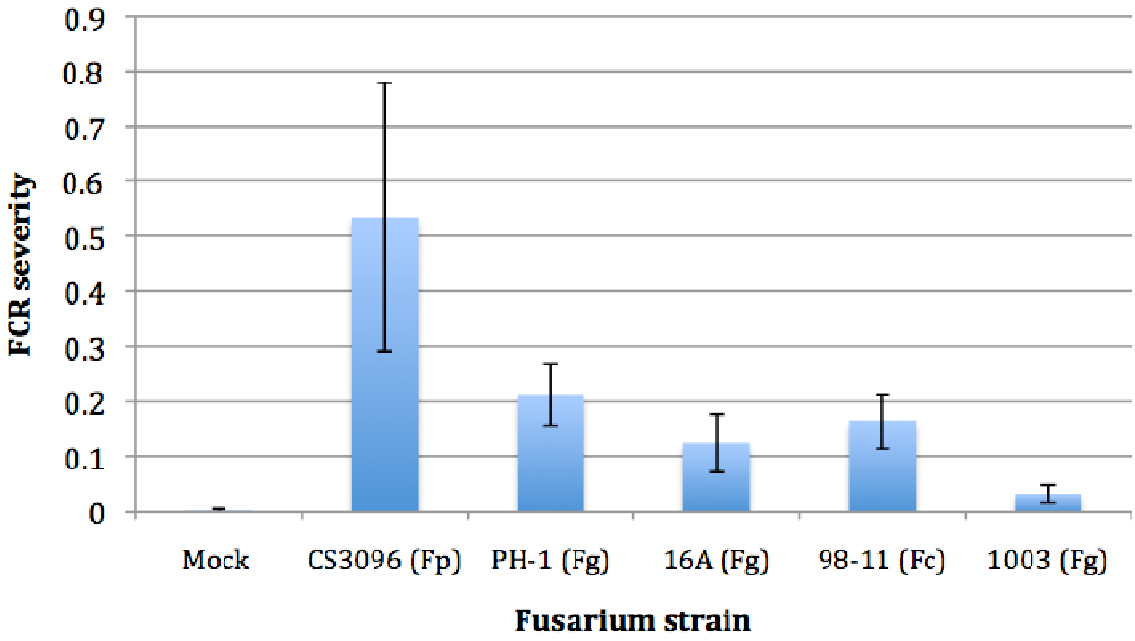


Figure 5.1: FCR severity of wheat seedlings at 28 dpi as a result of infections with wild type *Fusarium*. All error bars are the standard error of the mean for 30 independent biological replicates.

FCR DISEASE SYMPTOM DEVELOPMENT CAUSED BY *FUSARIUM* MUTANT STRAINS

Eight mutant *F. graminearum* strains that showed reduced virulence during FHB of wheat were analysed, they were ΔTri5Fg, ΔTop1Fg, ΔNtl1Fg, ΔSnf1Fg, ΔHog1Fg and ΔFgl1Fg. There were also two ΔMap1Fg isolates, which were derived from two different parent strains (PH-1 and 16A). The mutant strains ΔTri5Fg, ΔTop1Fg, ΔNtl1Fg and ΔSnf1Fg were all derived from PH-1 (Figure 5.2A). Because ΔSnf1Fg had a conidiation defect, the inoculum for this mutant was mycelia accompanied by an inoculation of wild type PH-1 mycelia prepared in parallel as a control.

Compared to its corresponding wild type, there was a trend for ΔTri5Fg to induce less FCR disease symptoms but this was not strongly significant (*P-value* = 0.106). There was stringer evidence that ΔTop1Fg, ΔNtl1Fg and ΔSnf1Fg showed highly statistically significant reductions in visible symptoms compared to the wild type, with *P-values* of ΔTop1Fg = 0.003, ΔNtl1Fg = 0.011 and ΔSnf1Fg = 0.002.

Each *Map1* mutant elicited less severe FCR disease symptoms than their corresponding wild types with *P-values* of Δ*Map1Fg*(PH-1) = 0.001 and Δ*Map1Fg*(16A) = 0.042. In fact, FCR severity for these mutants were similar to that of mock inoculated seedlings (Figure 5.2B).

The mutant strains ΔHog1Fg and ΔFgl1Fg were derived from the wild type *F. graminearum* strain 1003 which itself produced low levels of FCR disease (Figure 5.2C). Compared to their wild type, neither of these mutants showed a significant decrease in their ability to produce visible symptoms during FCR on wheat. On average, ΔHog1Fg induced similar levels of disease to the wild type (*P-value* = 0.394). Interestingly, the FCR severity score for ΔFgl1Fg was not lower than that of the wild type as it was during FHB infection. In fact, there was a slight increase in disease severity that was close to being statistically significant, with a *P-value* = 0.059.

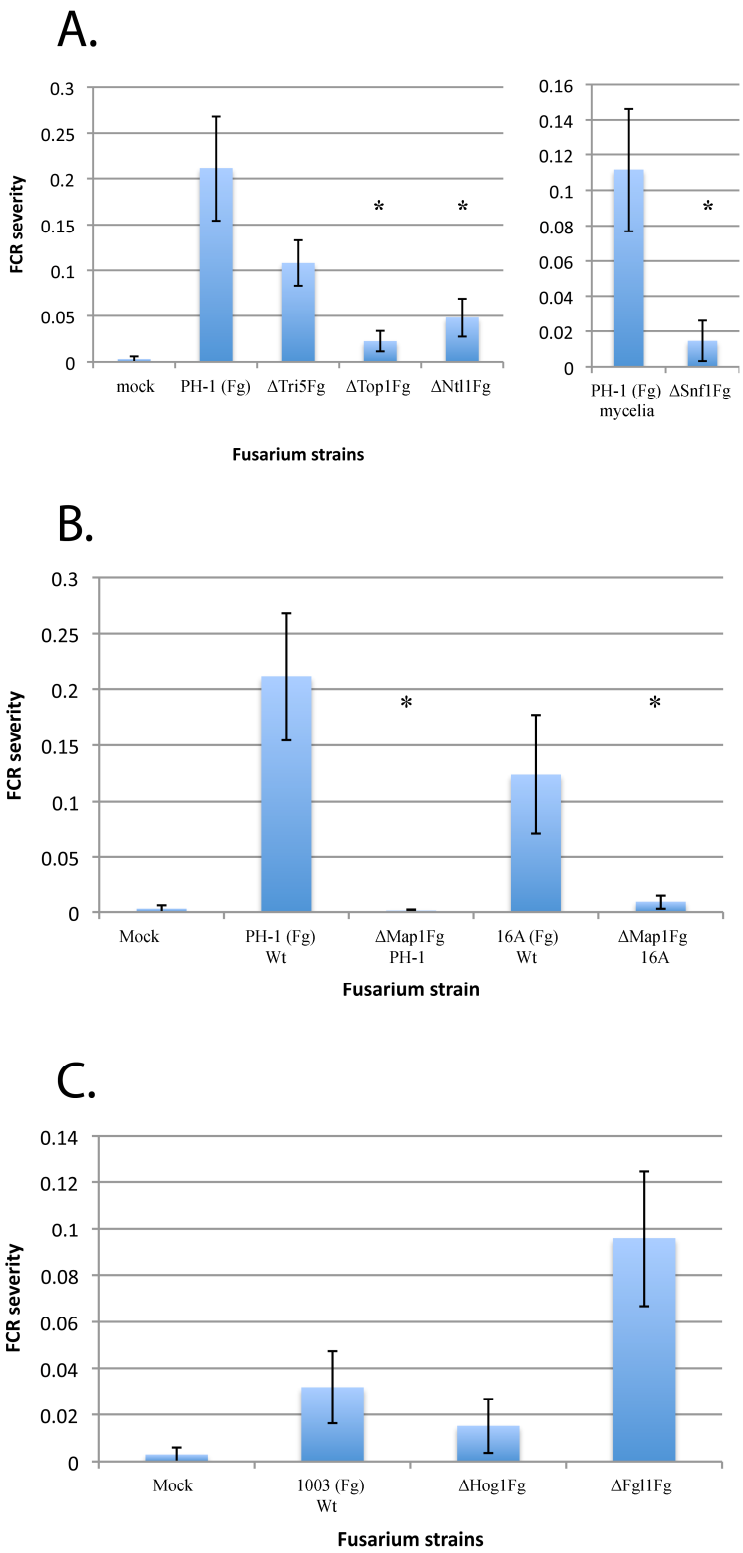


Figure 5.2: FCR severity of wheat seedlings at 28 dpi following infection with *F. graminearum* (A) PH-1 wild type and PH-1-derived mutants (B) *F. graminearum Map1* mutants and their corresponding wild types (C) *F. graminearum* 1003 wild type and 1003-derived mutants. All error bars are the standard error of the mean for 30 independent biological replicates.

FUNGAL BIOMASS MEASUREMENTS OF *FUSARIUM* WILD TYPE AND MUTANT ISOLATES DURING FCR DEVELOPMENT OF WHEAT.

Assigning a disease severity score based on visible symptoms and plant height is one method of assessing a FCR infection, but there are also other aspects of the disease that can be assessed to determine the degree of infection. To gain a better overall picture of the role each gene plays in FCR, the amount of fungal biomass generated during each infection was measured. This can test if the amount of biomass the fungus generates in an infection corresponds to the degree of visual symptoms observed. To do this, samples were freeze-dried after visible symptoms were assessed and DNA was extracted and fungal biomass was estimated by real time qPCR of genomic DNA as in Chapter 2.

Of the wild type *Fusarium* isolates, *F. pseudograminearum* and *F. graminearum* PH-1 produced the most fungal biomass during the crown rot infection (Figure 5.3). The average amount of biomass produced by both these isolates was similar, although *F. pseudograminearum* showed greater variability between samples. The *F. culmorum* (98-11) infection produced the next largest amount of fungal biomass, followed by *F. graminearum* 16A and *F. graminearum* 1003 which showed less biomass accumulation.

Gene deletion mutant strains ΔTop1Fg, ΔNtl1Fg and ΔSnf1Fg exhibited patterns in fungal biomass production similar to that seen for FCR severity scores (Figure 5.4A) and all 3 mutant strains had much lower fungal biomass compared to the parent isolate *F. graminearum* PH-1. Corresponding *P-values* were: ΔTop1Fg = 0.009, ΔNtl1Fg = 0.012 and ΔSnf1Fg = 0.330. The latter *P-value* was not statistically significant because of substantial variation in the biomass levels observed in the mycelial inoculation assay but there was virtually no fungal biomass detected in the ΔSnf1Fg inoculated plants and the overall trend was consistent with the symptom data. However, for ΔTri5Fg there was a strongly significant reduction in fungal biomass (*P-value* = 0.006), whereas only a trend of reduced visual symptoms with a higher *P-value* (0.106) was observed. Therefore, the fungal biomass measurements indicate that the deletion of *Tri5* affected fungal colonisation during FCR development, which was similar to the role of *Tri5* fungal colonisation in FHB.

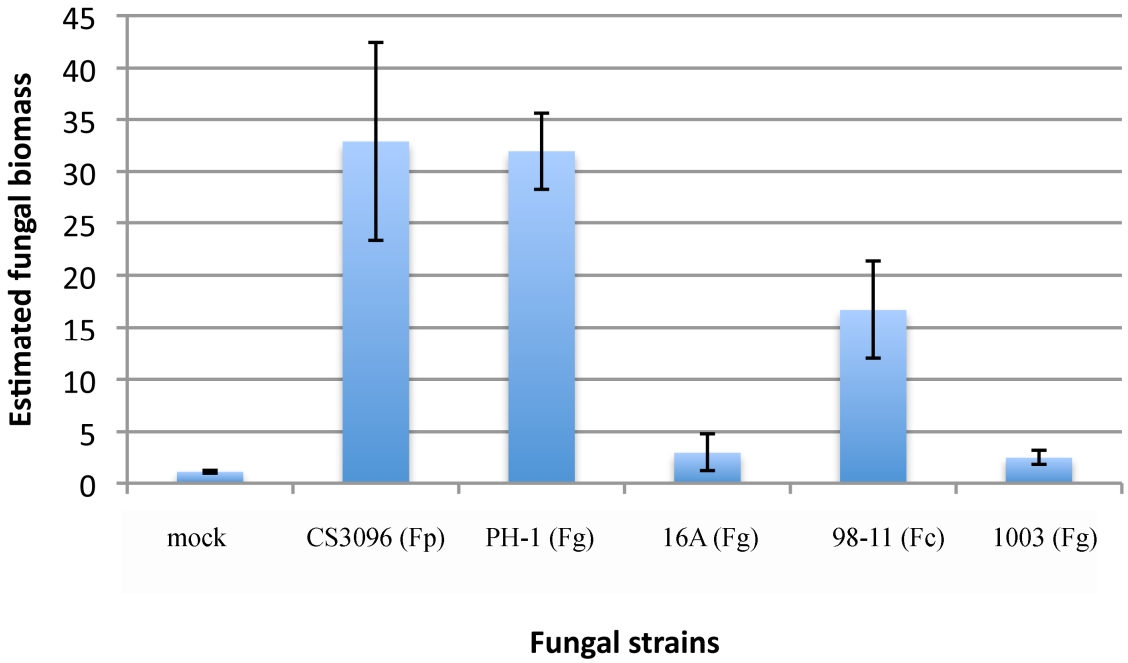


Figure 5.3: Estimation of fungal biomass accumulation in wheat seedlings during FCR infection with various wild type *Fusarium* species. All error bars are the standard error of the mean for three independent biological replicates.

Of the mutants that were derived from the English wild type isolate *F. graminearum* 1003, ΔHog1Fg developed a lower average of fungal biomass compared to the wild type but with weak significance (*P-value* = 0.092) (Figure 5.4B). FCR severity caused by the mutant ΔFgl1Fg scored higher than the wild type suggesting that it was more virulent. This trend was also observed in the fungal biomass estimations but there was considerable variation present and the difference from the wild type was not statistically significant (*P-value* = 0.281).

Both the *Map1* mutants tested in this experiment elicited less visible symptoms than their corresponding wild types and the differences were statistically significant. The amount of ΔMap1Fg(PH-1) biomass was also less than that of the PH-1 wild type and the difference was statistically significant (*P-value* = 0.013) as shown in Figure 5.4C. However, this was not the case for ΔMap1Fg(16A) which although on average did show lower biomass accumulation, this was not statistically significant (*P-value* = 0.340).

For ease of reading all of mutants showing significant differences in FCR severity and fungal biomass when compared to its respective wild type strain are summarised in Table 5.1.

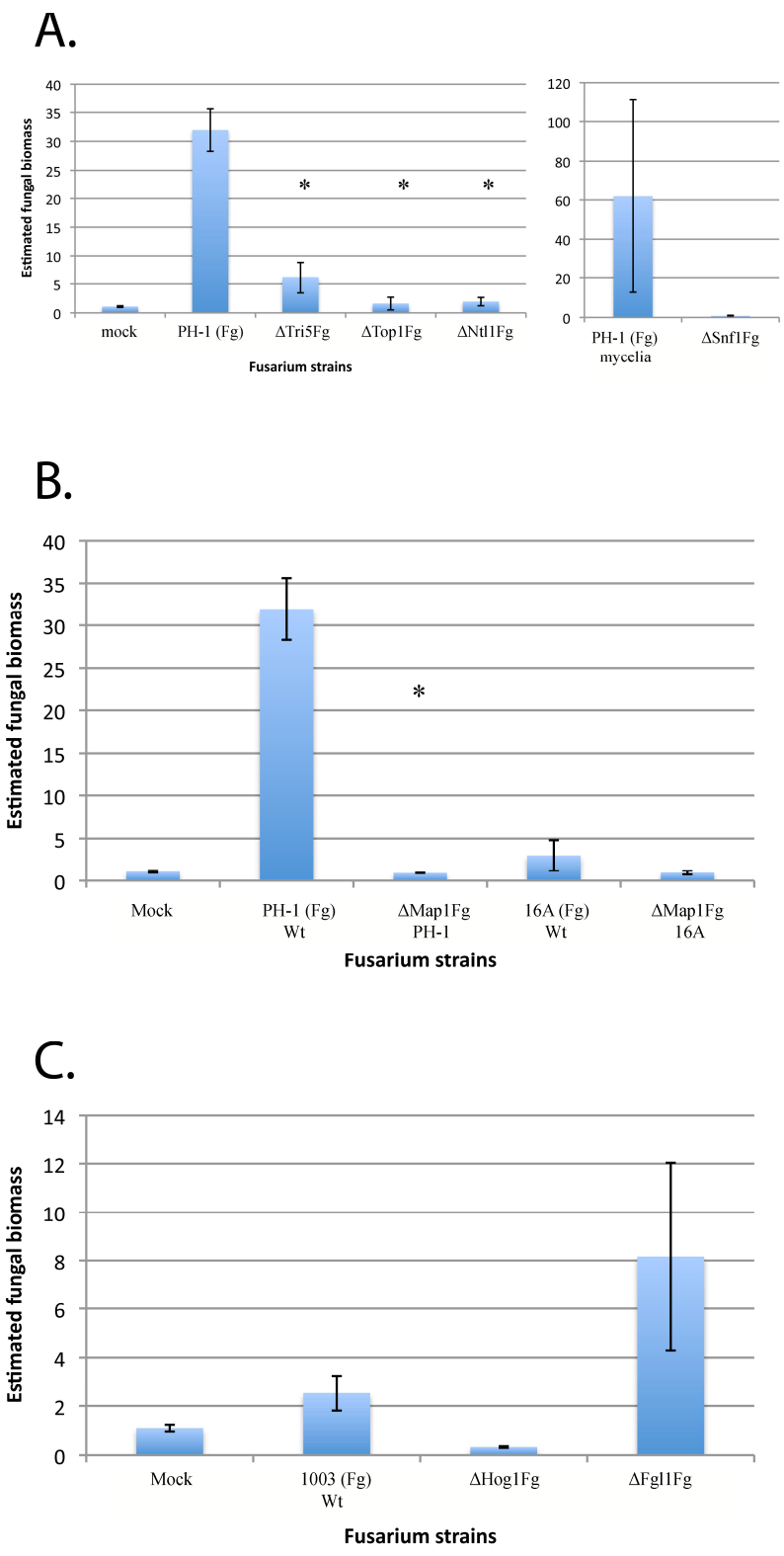


Figure 5.4: Estimation of fungal biomass accumulation in wheat seedlings during FCR infection by (A) *F. graminearum* PH-1 wild type and PH-1-derived mutants (B) *F. graminearum Map1* mutants and their corresponding wild types (C) *F. graminearum* 1003 wild type and 1003-derived mutants. All error bars are the standard error of the mean for three independent biological replicates.

Table 5.1: Summary of mutants showing significant differences in FCR severity and fungal biomass when compared to its respective wild type strain. Significant differences are represented with a √ followed by the *P-value*.

Fungal mutant name	Significant for FCR severity (*P-value*)	Significant for Fungal biomass (*P-value*)
ΔTri5*Fg* (PH-1)	------	√ (0.006)
ΔMap1*Fg* (PH-1)	√ (0.001)	√ (0.013)
ΔMap1*Fg* 16A	√ (0.042)	------
ΔTop1*Fg* (PH-1)	√ (0.003)	√ (0.009)
ΔNtl1*Fg* (PH-1)	√ (0.011)	√ (0.012)
ΔSnf1*Fg* (PH-1)	√ (0.002)	------
ΔHog1*Fg* (1003)	------	------
ΔFgl1*Fg* (1003)	------	------

Across all the samples tested in this study, there was a significant correlation (*P-value* = <0.05) between the amount of *F. graminearum* biomass detected and the crown rot severity score (Figure 5.5).

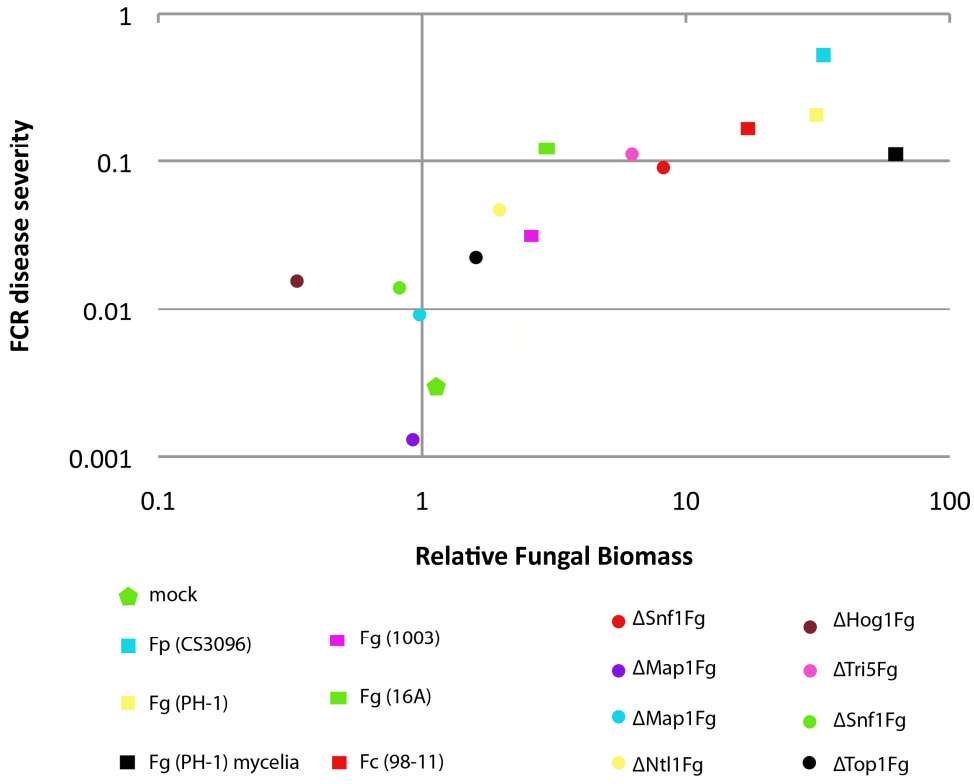


Figure 5.5: Correlation between fungal biomass and FCR disease severity (*R value = 0.56*)

Discussion

The aim of this chapter was to assess whether genes known to encode virulence factors in FHB could also be involved in fungal virulence during FCR. These two *Fusarium* diseases are composed of complex fungal-plant interactions that have evolved over a long time and therefore several aspects need to be considered when assessing disease severity. In this study, two criteria were evaluated to determine the degree of virulence of the *Fusarium* wild type and mutant strains. The two factors measured were visible symptom development and fungal biomass accumulation in wheat seedlings at 28 days post inoculation. The production of visible symptoms may be the result of both the host response to infection as well as the degree of fungal colonisation whereas the measurement of fungal biomass only estimates the level of fungal colonisation. However, both of these criteria may be important in disease development and its ultimate impact on plant performance and yield.

COMPARISON OF VISIBLE SYMPTOMS AND BIOMASS ESTIMATIONS OF DIVERSE *FUSARIUM* STRAINS

Of all wild type *Fusarium* isolates studied, the Australian *F. pseudograminearum* was the most virulent during crown disease. *F. pseudograminearum* exhibited a degree of variance between biological replicates in tests but on average produced the greatest amount of visible symptoms and together with the PH-1 *F. graminearum* isolate, accumulated the most fungal biomass during the infection. This was not surprising as the *F. pseudograminearum* strain used was originally isolated from FCR infected wheat and *Fusarium pseudograminearum* is also the chief FCR pathogen in Australia. However, it was interesting to observe that some other strains of *F. graminearum* isolated from infected heads were also capable of causing FCR disease symptoms. Quite noteworthy was that PH-1, the reference strain for genome sequencing and FHB studies in the USA produced as much fungal biomass as *F. pseudograminearum* during FCR, yet the PH-1 isolate caused less visible disease with similar levels as *F. graminearum* 16A and *F. culmorum* 98-11. These results indicate that *F. graminearum* strains isolated from infected heads overseas have an inherent capability to cause to FCR disease but vary considerably in their aggressiveness. This agrees with the findings of other researchers where similar cross-infectivity of Australian *Fusarium* isolates from FHB and FCR infections has been observed (Akinsanmi *et al*., 2004). Similarly, other strains of *F. graminearum* from FHB-infected wheat in the USA have been shown to cause FCR

using different inoculation procedures to those used here (Mudge *et al*., 2006). The reasons underlying variation in the aggressiveness of diverse *Fusarium* strains is not known but further understanding of the fungal genes involved in FCR disease would assist studies of fungal aggressiveness in the future.

COMPARISON OF VISIBLE SYMPTOMS AND FUNGAL BIOMASS OF MUTANT AND WILD TYPE *FUSARIUM* DURING FCR

Compared to the wild type, ΔTri5Fg displayed significantly less fungal biomass, but not a statistically significant reduction in visible symptoms. This indicates that DON production may have a function in the colonisation of plant tissue during FCR but may not play a major role in determining the extent of visible necrosis. This suggests that there must be fungal derived triggers other than trichothecene mycotoxins that cause the visible host necrosis in FCR infections.

Previous work has also shown that there is little or no change in visible FCR symptom development between DON- mutants with deleted *Tri5* genes and wild type in a stem base infection (Mudge *et al*., 2006). This study used re-isolation frequency to assess fungal colonisation of both inoculated and distal non-inoculated tissues of wheat during FCR disease (Mudge *et al*., 2006). This work also showed that the *TRI5* mutant was impaired in its ability to colonise upper nodes and the flag leaf of plants inoculated at the stem base. The more precise measurement of fungal biomass by real-time quantitative PCR used here indicates that the reduced ability of the non-DON producing mutants to colonise the plant may reflect a reduced net mass of fungus at the point of infection. In many respects the impact of the deletion of the *Tri5* gene on FCR has several parallels with its impact on FHB. In FHB disease, DON is required for the fungus to spread from the point of inoculation down the rachis and through the spike but not for initial infection (Jansen *et al*., 2005, Proctor *et al*., 1995), while in FCR it also appears to be necessary for fungal colonisation and spread up the stem. It is therefore possible that DON is necessary to impair plant cell defence function and provide nutrients that can support fungal colonisation. The effect of DON on host cells is considered further in Chapter 6.

Both ΔTop1Fg and ΔNtl1Fg had statistically significant reductions in symptoms and fungal biomass. Both of these genes have been shown to be necessary for the full expression of FHB symptoms (unpublished work by Andrew Beacham and Thomas Baldwin at Rothamsted Research).

ΔTop1Fg carries a deletion of a gene encoding a putative subunit of the anaphase-promoting complex (APC) and therefore could be involved in the *Fusarium* cell cycle and separation of sister chromatids. The APC cyclosome complex can still function without this subunit but not at optimum levels and consequently this may also slow down fungal cell division during infection leading to less visible symptoms as a direct result of less fungal biomass. Alternatively, reduced growth may provide more scope for the host to respond with defences that constrain the fungus and disease development. However it is clear that this gene is required for both FHB and FCR development.

ΔNtl1Fg carries a deletion of a gene encoding a probable neutral trehalase involved in the metabolism of trehalose. This gene is the only putative neutral trehalase in the *F. graminearum* genome so presumably the mutant could accumulate trehalose but was unable to metabolize it. Trehalose is a sugar that accumulates in fungal cells and has high water retention, thus can protect against desiccation and can act as an energy store during times of stress (Abrashev *et al.*, 2008, Attfield, 1987, Lowe *et al*, 2009) . We have seen in Chapter 2 that during phase 1 *F. graminearum* colonises the surface of the plant stem which may be a nutrient-poor environment thus requiring survival mechanisms such as that involving neutral trehalase which would be required to remobilize trehalose for energy.

ΔSnf1Fg carries a deletion of a gene that encodes a probable serine/threonine protein kinase. Loss of this protein caused statistically significant reduction in visible symptoms (*P-value* = 0.012) but not accumulation of fungal biomass (the latter was largely due to variation in the wild type biological replicates). Knockout of this gene has been shown to affect the metabolism of some carbohydrates including galactose, sucrose and trehalose and also spore production and development (Lee *et al.*, 2009). If the *Snf1* gene regulates the production of enzymes that metabolize sugars, starch and plant cell wall components it may be important for obtaining enough plant derived nutrients to establish an infection. Also the presence of these cell wall degrading enzymes and/or the by-products of the metabolized carbohydrates may be triggers for plant defences and necrotic lesion development.

The *Map1* gene proved to be essential for *Fusarium* virulence during crown rot on wheat. It is believed that *Map1* controls a range of important biological functions that include the formation of sexually produced perithecia (Urban *et al.*, 2003) and overall induction of cell-wall degrading enzymes (Jenczmionka & Schafer, 2005). Results show that one or more of these functions are

essential in producing both visible symptoms in FCR and also the production of fungal biomass through colonisation. Obviously the results obtained here for FCR support the notion that *Map1* is a central regulatory gene vital for fungal pathogenesis.

Neither ΔHog1Fg nor ΔFgl1Fg were statistically significantly different from the wild type for either visible symptoms or fungal biomass. However, both of these mutants were derived from the wild type strain 1003 which elicited very low levels of FCR development compared to the other strains. This made it difficult to determine whether differences observed in the mutant strains were statistically significant with the number of replicates used in this experiment. On average ΔFgl1Fg showed both higher levels of visible symptoms and fungal biomass than the wild type and was the only mutant isolate tested that showed this trend. *Fgl1* is therefore only a virulence factor for FHB but not during FCR. *Fgl1* encodes a probable triacylglycerol lipase precursor that is most likely involved in metabolism of lipids. This lipase may be useful during FHB because it can metabolize the triacylglycerol lipids present in the developing wheat grain, compared to the leaf sheaths during early stages of colonisation. Leaf sheaths, although rich in membrane phospholipids are less likely to contain storage lipid droplets. However, for greater certainty on the role of the *Fgl1* and *Hog1* genes during FCR it is recommended that these mutations be recreated in a strain of *F. graminearum* that has greater aggressiveness for FCR such as PH-1. This would provide higher disease ratings in the wild type and greater resolution for assessing reductions or increases in virulence in the mutants.

Comparisons of individual mutants indicated that in some instances there was not a direct relationship between symptom development and fungal biomass in the FCR infected tissues. However, when all the data across all the experiments conducted in this study were plotted a significant relationship between symptom development and fungal biomass was observed suggesting that in general these two parameters of infection are linked.

COMPARISON OF FUNGAL GENES REQUIRED FOR FHB AND FCR DISEASES

The results of this study show that *Tri5* is not essential for visible FCR symptoms, but does play a role in the colonisation of the stem base and therefore can be classified as a colonisation factor. The genes *Top1*, *Nth1* and *Snf1* and their role in FHB disease development have not yet been published but mutants lacking these genes have been reported as having reduced virulence during

FHB (Hammond-Kosack, Urban, Beacham and Baldwin personal communication). Reduced virulence was also observed in FCR infections. *Map1* was the only gene tested here where the mutant showed a loss of pathogenicity in FHB and this was also the case for crown rot. This demonstrates that the regulatory role this gene plays is fundamental to *F. graminearum* pathogenicity irrespective of which disease it is involved in.

Fgl1 and *Hog1* have been reported to be virulence factors in FHB (Voigt *et al*., 2005, Ramamoorthy *et al*., 2007). However, this classification may not hold true for FCR. On average ΔFgl1Fg exhibited signs of increased disease symptom development with reasonable significance (*P-value* = 0.059) and ΔHog1Fg did not show any statistically significant difference in virulence from the wild type for FCR development. *Hog1* has a similar transcription profile during FCR and FHB, but *Fgl1* transcription differs slightly in FCR and FHB. During FCR, *Fgl1* did not show a significant change in expression throughout the infection whereas during FHB its transcription significantly increased during the first two days before reaching a plateau (7-fold induction of transcription from vegetative mycelia to 3 dpi). However these interpretations should only be considered preliminary and need to be verified using a parental strain that has higher aggressiveness for FCR.

Together with *FgABC1* and *FgSOD1*, the above virulence genes can be placed on a Venn diagram that starts to illustrate the molecular infection program *F. graminearum* utilizes during both FCR and FHB (Figure 5.6). The information gathered in this study will be important when developing biological controls for *Fusarium* disease of wheat. By investigating genes that play a role in one of the *Fusarium* wheat diseases but not the other we can begin to understand how *F. graminearum* has evolved to be able to incite the two types of disease. However, the targeting of virulence and pathogenicity genes that make up the core program of *Fusarium* infection and that are important for both FHB and FCR, gives a larger scope for controlling *Fusarium* diseases in different environments where either head blight or crown rot can be more prevalent.

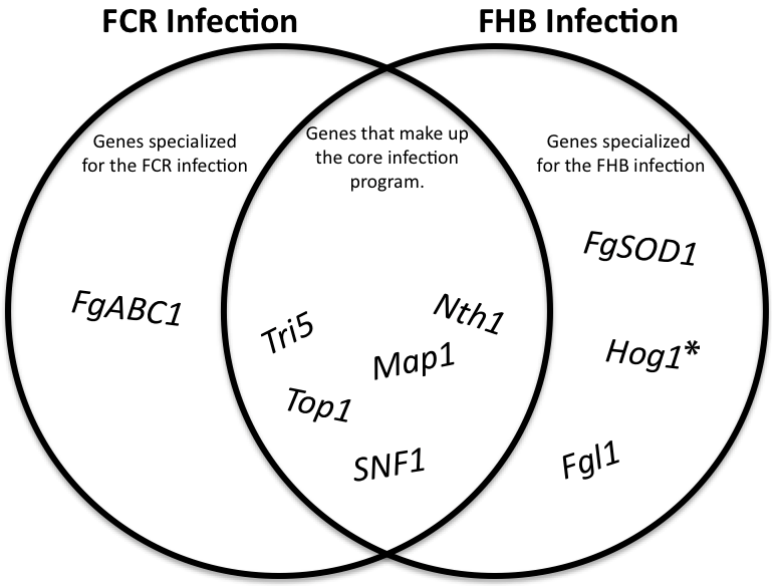


Figure 5.6: Venn diagram showing the genes important for the infection programs of FHB, FCR or both. * indicates placement based on preliminary results and requires confirmation in a more aggressive strain for FCR.

Experimental Procedures

FUNGAL STRAINS AND INOCULUM PREPARATION

FCR severity scores for all wild type and mutant isolates were obtained through experiments completed in collaboration at Rothamsted Research (Harpenden, England). These experiments were conducted with,

Wild type isolates:

PH-1 (Fg): Sequenced North American isolate of *Fusarium graminearum*

CS3096 (Fp): Australian *Fusarium pseudograminearum* isolate

1003 (Fg): English *Fusarium graminearum* isolate

16A (Fg): German *Fusarium graminearum* isolate

98-11 (Fc): English *Fusarium culmorum* isolate

Mutant isolates:

ΔTri5*Fg*: *Tri5* deletion mutant of PH-1 (Proctor *et al*., 1995)

ΔMap1*Fg* *Map1* deletion mutant of PH-1 (Jenczmionka *et al*., 2003)

ΔTop1*Fg* *Top1* deletion mutant of PH-1 (unpublished Dr Thomas Baldwin)

ΔNtl1*Fg* *Ntl1* deletion mutant of PH-1 (unpublished Andrew Beacham)

ΔSnf1*Fg* *Snf1* deletion mutant of PH-1 (unpublished Dr Martin Urban)

ΔHog1*Fg* *Hog1* deletion mutant of PH-1 (Ramamoorthy *et al*., 2007)

ΔMap1*Fg* *Map1* deletion mutant of 16A (Urban *et al*., 2003)

ΔFgl1*Fg* *Fgl1* deletion mutant of PH-1 (Voigt *et al*., 2005)

For inoculation, macroconidia were produced by inoculating SNA minimal nutrient agar plates with a half-strength potato dextrose agar (PDA) plug colonised with the aforementioned fungal strains, then incubating at room temperature for 7 days. Then 2 mL of T3 broth was added and the plate was incubated for a further 2 days. Then spores were washed off the PDA surface with sterile water and the spore concentration was adjusted to $1x10^6$ spores per mL in distilled water. Spore preparations used were fresh for all inoculations.

PLANT GROWTH, INOCULATION AND HARVESTING TECHNIQUES

FHB-susceptible wheat cultivar Bob White was used for all FCR assays, seedlings were grown in a controlled environment facility with day-time conditions of 24^{o}C temperature with 60% humidity and night-time conditions of 15^{o}C with 90% humidity. Trays of plastic seedling punnets (5×5 cm per punnet, 30 punnets per tray) were filled with sterile soil mix comprising 50% sand and 50% peat v/v and two seeds were planted in each punnet. Seedlings were grown and inoculated at the base of the shoot 14 days after planting as described by (Mitter et al., 2006b). Bob White seedlings were inoculated with corresponding fungal strains and were harvested at 28dpi. 10 seedlings were pooled for a biological replicate and there were three biological replicates per fungal strain. Visible symptoms were scored as described below for all seedlings then infected tissue was harvested from each seedling by excising the shoot tissue from the crown to leaf 1 with a pair of sharp scissors. The infected tissue was then freeze-dried and used for fungal biomass analysis at the CSIRO Plant Industry, St Lucia, Australia.

SCORING FCR DISEASE SEVERITY

Wheat seedlings were inoculated and rated for FCR using the same method as in Chapter 3. For each *F. graminearum* isolate 30 seedlings were inoculated for independent biological replicates.

DNA EXTRACTIONS AND *FUSARIUM* BIOMASS ESTIMATIONS

Freeze-dried shoot bases were ground using metal balls with vigorous shaking and genomic DNA was extracted using a QIAGEN DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was eluted into 100 µL of sterile water and stored at -20 ^{o}C until needed. *F. graminearum* biomass was estimated indirectly using real-time quantitative PCR (RT-qPCR). PCR reactions were performed as described in Chapter 2.

CHAPTER 6: Investigation of the role of trichothecene in host defence induction during crown rot of wheat.

Introduction

In Chapter 5, it was shown that the trichothecene synthase gene *Tri5* may have a role in *F. graminearum* virulence during FCR of wheat. It was observed that a deletion of the *Tri5* gene in the *F. graminearum* strain PH-1 did not reduce the ability of the fungus to cause visible disease symptoms, but did significantly reduce the ability of the fungus to proliferate and colonise the wheat plants, thus this gene was referred to as a colonisation factor. In FHB it has also been found that DON is required for *F. graminearum* to spread from spikelet to spikelet on the wheat head (Jansen *et al*., 2005, Proctor *et al*., 1995). In this chapter, the effects of a DON non-producing *F. graminearum* on FCR was studied further with all experimental work being carried out in Australia with local fungal isolates.

The work described in this chapter was included as a contribution to the publication Desmond *et al.,* (2008b) which is included in this chapter. Data contributed to this paper from this thesis pertained to the differential ability of the wild type and *Tri5* mutant *F. graminearum* to accumulate biomass and the effect of the *Tri5* mutation on expression of host defence genes. The work is included in the Results section of Desmond *et al.,* (2008b) under the heading '*differential defence gene induction by a DON-non-producing fungal strain at late stages of CR disease development*'. On page 110 of this thesis and page 438 of the following paper.

Previous work by Dr. Olivia Desmond had shown that both DON treatment and inoculation by *F. pseudograminearum* and *F. graminearum* led to the induction of a suite of plant defence genes. This led to the hypothesis that perhaps it was the release of the trichothecene DON that triggered the induction of host defence genes during infection. To test this hypothesis, an experiment was conducted in which a *Tri5* mutant of an Australian *Fusarium* isolate was used to inoculate wheat stem bases and DNA and RNA samples were taken over a time course to measure fungal biomass and host defence transcripts, respectively. The outcome, described in the attached publication was that host defences are induced by both the wild type and the *Tri5* mutant. This indicates that DON is not responsible for host defence induction. The only difference in expression of host defence genes observed was at the latter stage of infection (28 dpi) where plants inoculated with the *Tri5* mutant showed reduced expression of host defence genes. This was also the only stage of FCR infection when a difference in fungal biomass was observed between wild type and mutant suggesting that other factors may also be responsible for the difference in host defences.

In summary, these findings were consistent with those of Chapter 5. There was little effect of the *Tri5* mutant on the host response, measured either as visible symptoms or as defence gene expression in this chapter. However, the mutation does affect fungal colonisation at late stages of inoculation further implicating the importance of trichothecene toxins in fungal colonisation.

The characterisation of the *Tri5* mutant reported in the attached paper is described in Appendix 3.

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The *Fusarium* mycotoxin deoxynivalenol elicits hydrogen peroxide production, programmed cell death and defence responses in wheat

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SUMMARY

Fusarium species infect cereal crops worldwide and cause the important diseases Fusarium head blight and crown rot in wheat. *Fusarium* pathogens reduce yield and some species also produce trichothecene mycotoxins, such as deoxynivalenol (DON), during infection. These toxins play roles in pathogenesis on wheat and have serious health effects if present in grain consumed by humans or animals. In the present study, the response of wheat tissue to DON has been investigated. Infusion of wheat leaves with DON induced hydrogen peroxide production within 6 h followed by cell death within 24 h that was accompanied by DNA laddering, a hallmark of programmed cell death. In addition, real-time PCR analysis revealed that DON treatment rapidly induced transcription of a number of defence genes in a concentration-dependent manner. Co-treatment with DON and the antioxidant ascorbic acid reduced these responses, suggesting their induction may be at least partially mediated by reactive oxygen species (ROS), commonly known to be signalling molecules in plants. Wheat defence genes were more highly expressed in wheat stems inoculated with a DON-producing fungal strain than those inoculated with a DON-non-producing mutant, but only at a late stage of infection. Taken together, the results are consistent with a model in which DON production during infection of wheat induces ROS, which on the one hand may stimulate programmed host cell death assisting necrotrophic fungal growth, whereas, on the other hand, the ROS may contribute to the induction of antimicrobial host defences.

INTRODUCTION

Many plant pathogenic fungi produce secondary metabolites, including mycotoxins, during the infection process. Mycotoxins, such as the trichothecene deoxynivalenol (DON) produced by some *Fusarium* species, are not always essential for initiating disease but are often linked with increased aggressiveness (Desjardins *et al.*, 1995). The effect of DON on cell function has been most extensively studied in animal cells in attempts to understand its toxicity (Pestka *et al.*, 2004; Rocha *et al.*, 2005). These studies have resulted in a model where trichothecenes inhibit protein synthesis by binding to the 60S ribosomal subunit, activating a cell signalling programme that results in apoptosis. In conjunction with this, there is an induction of cytokine-regulated gene expression resulting in an inflammatory response. In macrophages, as well as T and B cells of the mammalian immune system, these complex effects mean that trichothecenes such as DON can be either immunostimulatory or immunosuppressive depending on dosage, duration and frequency of exposure (Pestka *et al.*, 2004).

Fusarium head blight (FHB) and crown rot (CR) are both diseases of wheat caused by several *Fusarium* species, including *F. graminearum* (*Fg*) and *F. pseudograminearum* (*Fp*) (Akinsanmi *et al.*, 2004). The major difference between these diseases is the site and timing of infection, with FHB infecting wheat heads around the time of anthesis, and CR infecting stem base and crown tissue at all growth stages. DON is produced during both FHB and CR diseases (Blaney and Dodman, 2002; Mudge *et al.*, 2006) and this is a major concern due to its toxicity to humans and animals following consumption of contaminated grain (Larsen *et al.*, 2004).

The role of DON and other trichothecene mycotoxins during pathogenesis has been analysed using mutant fungal strains that do not produce toxin (Hohn and Desjardins, 1992). Studies on a strain of *Fg* that has a mutation in the *Tri5* gene encoding a DON biosynthetic enzyme have shown that *Fg* strains unable to produce DON are less aggressive during FHB in both wheat and barley (Boddu *et al.*, 2007; Langevin *et al.*, 2004). More specifically in wheat, the mycotoxin appears to be necessary for fungal passage from infected florets into the rachis from where it can further colonize the head (Jansen *et al.*, 2005). These DON-non-producing *Fg* strains are unable to prevent thickening of host cell walls after penetration and so their movement from the point

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of infection is hindered (Bai *et al.*, 2002; Jansen *et al.*, 2005; Maier *et al.*, 2006). There is also evidence that FHB-resistant wheat genotypes accumulate far less DON than susceptible ones (Goswami and Kistler, 2005; Wilde and Miedaner, 2006). For FHB, it therefore appears that occurrence of disease may not be dependent on the toxin, but DON production does affect disease levels, and so improving resistance to DON may improve resistance to the disease. The role of DON during CR is less well defined than for FHB, but it appears to be necessary for fungal colonization of upper stem nodes following infection at the crown and stem base (Mudge *et al.*, 2006).

Several studies have shown that fungal derived toxins can elicit responses in plants that have aspects in common with well-known pathogen-induced responses. It has recently been reported that another trichothecene produced by some *Fusaria*, the *T*-2 toxin, induces hydrogen peroxide (H_2O_2) production, inhibits protein synthesis and stimulates cell death in the non-host plant *Arabidopsis thaliana* at concentrations as low as 0.4 mg/L (Nishiuchi *et al.*, 2006). This study also showed that DON can induce defences in *A. thaliana* plants but only at the higher concentration of 30 mg/L. In barley, a comparison of gene expression induced by wild-type and a DON-non-producing strain of *F. graminearum* revealed that DON is responsible for the induction of genes encoding proteins involved in ubiquitination and programmed cell death (PCD) among others (Boddu *et al.*, 2007). In wheat, differential display analysis has been used to demonstrate that DON affects transcript levels of a few specific host genes in roots, including peroxidase genes (Ansari *et al.*, 2007). However, the physiological and molecular responses of wheat cells to DON exposure have not been described.

We aimed to assess the effects of DON on wheat defence responses and observed the production of H_2O_2, a well-known signalling molecule, followed by cell death, a phenomenon frequently observed in mammalian cell lines exposed to DON (Pestka *et al.*, 2004). In addition, DON induced a range of well-known defence genes and interestingly a DON-producing fungal strain induced higher levels of defence transcripts than a DON-non-producing mutant during disease development. Both cell death and defence gene induction was reduced by co-treatment of DON and an antioxidant that would scavenge free radicals such as H_2O_2. These results suggest that DON produced during *Fusarium*-related diseases may play a role in the activation of wheat defence responses and cell death, at least partially via the signalling molecule H_2O_2.

RESULTS

DON elicits ROS production, genomic DNA laddering and cell death in wheat

Wheat stem tissue was infiltrated with a solution of 100 or 200 mg/L DON, resulting in the production of localized H_2O_2 microbursts (Levine *et al.*, 1994) within 6 h after treatment (Fig. 1A–C; Table 1; supplementary Fig. S1). Co-infiltration of DON with 40 mM ascorbic acid, an antioxidant capable of scavenging reactive oxygen species (ROS), or 4 mg/L cycloheximide, a eukaryotic protein synthesis inhibitor, reduced the amount of H_2O_2 produced (Table 1; supplementary Fig. S1). Trypan blue staining to reveal cell death was negative at this 6-h time point (data not shown). By 24 h post-infiltration, H_2O_2 staining was no longer visible in DON-infiltrated tissue (data not shown), but the tissue stained positively for cell death, primarily in stomatal guard cells and also in mesophyll cells of samples treated with 200 mg/L DON (Fig. 1D–F; Table 1; supplementary Fig. S1). Again, co-infiltration with ascorbic acid reduced cell death and co-infiltration with cycloheximide prevented cell death (Table 1; supplementary Fig. S1).

Table 1 Summary of H_2O_2 and cell death observed in infiltrated wheat leaf tissue stained with DAB 6 h after treatment and stained with trypan blue 24 h after treatment.

	H_2O_2—6 h	Cell death—24 h
Mock	–	+
100 mg/L DON	+++	++
200 mg/L DON	+++	++++
100 mg/L DON + 7 g/L AA	++	+
100 mg/L DON + 4 mg/L CHX	++	–

DON, deoxynivalenol; AA, ascorbic acid; CHX, cycloheximide. Scale: +, some stomata affected or sporadic H_2O_2 microbursts in some tissue; ++, most stomata affected or many H_2O_2 microbursts in some tissue; +++, all stomata affected or many microbursts in all tissue; ++++, widespread tissue affected including stomata and mesophyll cells. Further images of the tissue described in this table are shown in supplementary Fig. S1.

To investigate the mechanism behind DON-induced cell death further, genomic DNA collected from wheat stems 24 h after infiltration was analysed for cleavage products by gel electrophoresis. A shown in Fig. 1G, a distinct DNA laddering pattern was observed in DON-treated leaves when compared with mock-treated controls. Similar to cell death visualized using trypan blue staining, DNA laddering appeared to be reduced when tissue was co-infiltrated with ascorbic acid. This effect of ascorbic acid on DNA laddering was observed in two independent experiments. Visually quantifying differences between these DNA laddering profiles was subjective, and therefore to quantify the results, the total number of pixels in each lane was assessed using ImageJ software. This was then compared with the number of pixels in the region of the image showing only degraded DNA (< 5000 bp). This method of quantification showed that DNA from mock-treated samples was only 5% degraded, while that from DON-treated samples was 66% degraded; combining ascorbic acid with the DON treatment reduced degradation to 29%. DON-induced DNA laddering as observed here indicates

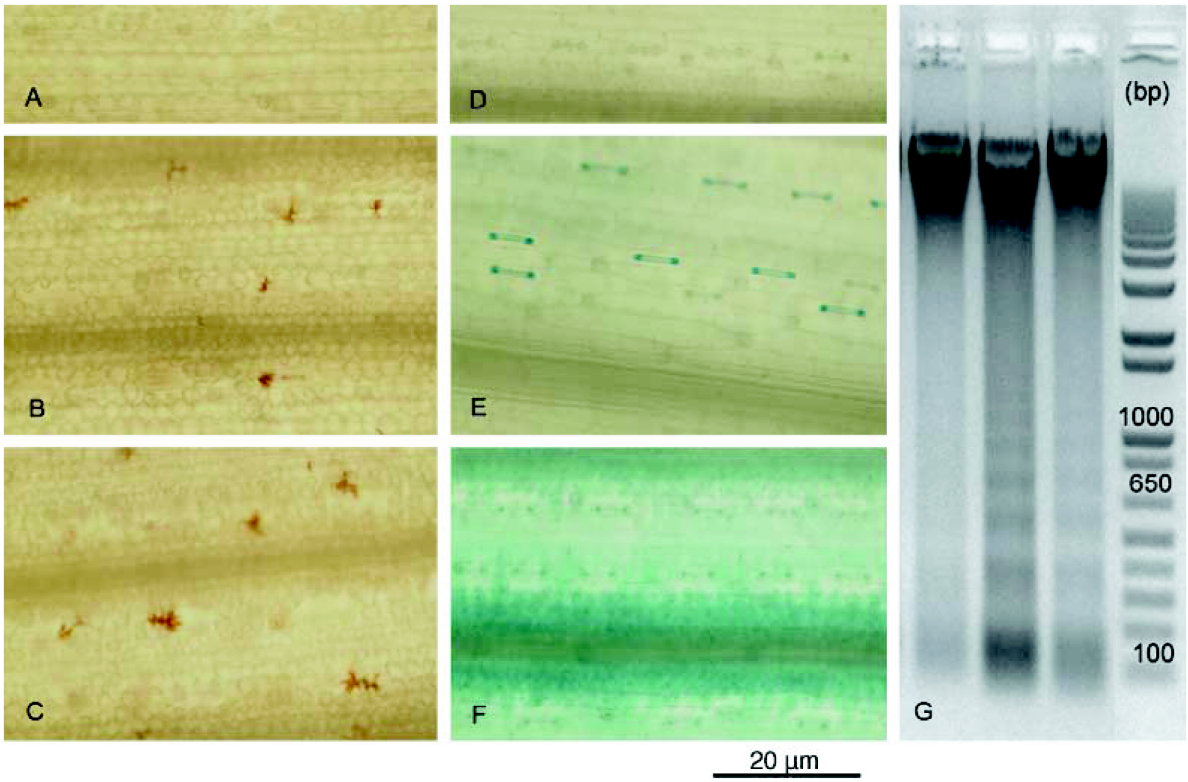

Fig. 1 Infiltration of wheat tissue with DON resulted in H_2O_2 production, cell death and DNA laddering. DAB-stained wheat leaf tissue from 2-week-old seedlings showing H_2O_2 production at 6 h after (A) mock infiltration, (B) 100 mg/L DON infiltration and (C) 200 mg/L DON infiltration. Trypan blue-stained wheat leaf tissue 24 h after (D) mock infiltration, (E) 100 mg/L DON infiltration and (F) 200 mg/L DON infiltration. (G) Genomic DNA laddering in wheat 24 h after infiltration with mock solution shown in the first lane, 100 mg/L DON shown in the second lane, 100 mg/L DON combined with 7 g/L ascorbic acid shown in the third lane and the 1-kb + DNA size ladder (Invitrogen) in the fourth lane. Further images of tissue from all infiltration treatments are shown in supplementary Fig. S1.

induction of a host-mediated PCD process (Ryerson and Heath, 1996).

Fp and *Fg* are closely related species that are both able to cause CR and FHB in wheat, and both pathogen species produce DON (Mudge *et al.*, 2006). Tissue inoculated with *Fp* spores was studied in detail and revealed H_2O_2 production and cell death, indicating defences induced by purified DON are consistent with those induced during a natural CR infection. DON has previously been shown to be produced during CR at levels > 100 mg/L when *Fp* is the causal agent (Mudge *et al.*, 2006). Within 7 days after inoculation of wheat, using a detached leaf assay, intense H_2O_2 production was observed in some stomatal guard cells that were in close proximity to *Fp* spores (Fig. 2A). DAB staining also revealed the presence of H_2O_2 in some fungal spores (Fig. 2A inset), a phenomenon previously associated with pathogenicity in other host–pathogen interactions (Egan *et al.*, 2007). Host cell death of *Fp*-inoculated wheat tissue was observed at multiple infection points and spread in a way that seemed to follow vascular tissue (Fig. 2B; supplementary Fig. S2). Stomatal guard cells showed the most intense and widespread staining for cell death among those cells surrounding infection points. Preliminary examination of *Fg* infection sites indicated similar host responses to those observed with *Fp* (data not shown).

Induction of wheat defence gene transcripts and proteins by DON

We have previously shown that drop inoculation of wheat with *Fp* spores leads to the induction of a suite of defence gene transcripts including *PR1.1*, *PR2* (β-*1–3 glucanase*), *PR3* (*chitinase*), *PR4* (*wheatwin*), *PR5* (*thaumatin-like protein*), *PR10*, *peroxidase* and *germin-like* (Desmond *et al.*, 2005). Herein, a similar drop treatment of wheat seedlings with a solution of DON also induced expression of these well-known defence genes. Figure 3A shows transcript induction by DON, generally in a concentration-dependent manner, within the range of 1–100 mg/L. Treatment

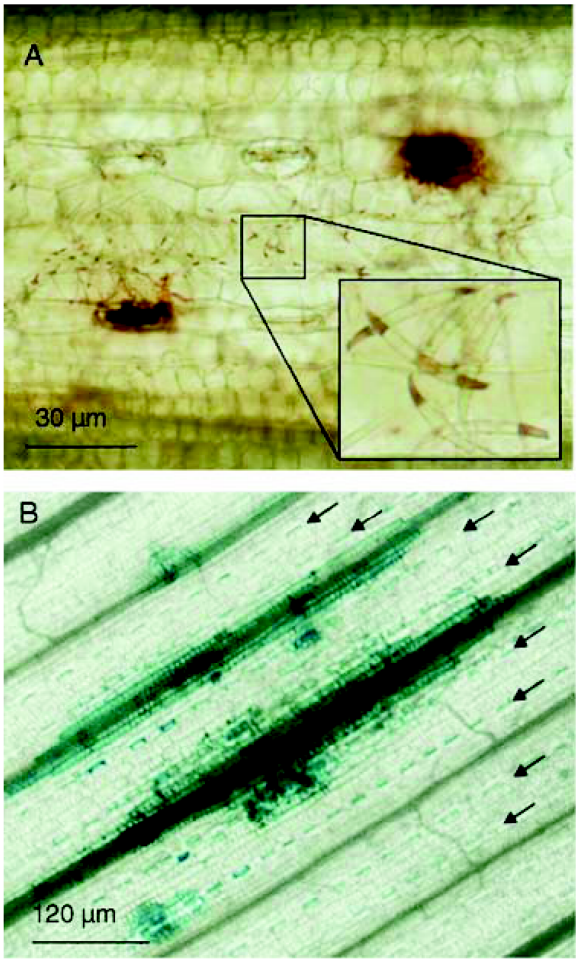

Fig. 2 H_2O_2 production and cell death was observed in wheat leaves after inoculation with *F. pseudograminearum* spores. (A) Wheat leaf tissue was inoculated using a detached leaf assay stained using DAB 7 days later. H_2O_2 was visible in stomata that were in close proximity to spores and also in the spore tips (inset). (B) Trypan blue staining of inoculated leaf tissue showed cell death seemed to follow vascular tissue and was most widespread in stomatal guard cells. Rows of affected stomatal guard cells that surround the primary infection site are indicated by arrows. Further images of cell death in inoculated tissue are shown in supplementary Fig. S2.

with 0.1 mg/L DON did not induce defence gene expression (data not shown). Interestingly, the most highly induced genes were *germin-like* and *peroxidase*, both genes implicated in reactive oxygen metabolism.

DON is known to be a potent inhibitor of eukaryotic protein synthesis at concentrations well below 100 mg/L (Nishiuchi *et al.*, 2006). Therefore, a Western blot to assess protein levels was performed on 100 mg/L DON-treated wheat samples using PR2- (β-1,3-glucanase) and PR3- (chitinase) specific antibodies. Again, ImageJ was used to quantify pixels for each band, and protein abundance as a percentage of Rubisco is shown for each band in Fig. 3B. PR2 and PR3 are clearly more abundant both visually and quantitatively in both *Fp*-inoculated and DON-treated samples compared with controls.

The influence of ROS on DON-induced defence gene induction was investigated by comparing samples treated with DON alone or DON combined with ascorbic acid. Figure 4 shows that the presence of ascorbic acid reduced the average induction of defence genes at both 6 and 24 h after treatment. Statistical analysis showed this reduction was significant ($P < 0.05$) for at least *PR3*, *peroxidase* and *PR2*.

Variation in the level of induction following 100 mg/L DON treatment was observed in gene expression analysis presented in Figs 3A and 4. The quantitative gene expression data obtained from RT-qPCR often shows variation in experiments set-up independently of each other. Slight variations in light conditions, temperature, inoculum quality and developmental stage of treated plants could not be completely prevented, and can affect gene induction during stress responses. Therefore, the question of whether gene induction was consistently observed would be more relevant than the actual fold-induction observed in each experiment. In the present case, induction was observed in all three biological replicates tested on both occasions that have been reported.

Differential defence gene induction by a DON-non-producing fungal strain at late stages of CR disease development

To test whether DON may play a role during CR infection of wheat, we assessed fungal biomass and the levels of defence gene transcripts in wheat stem tissue inoculated with either the transgenic DON-non-producing strain of *Fg* (*Fg* Δ*Tri5*) or its wild-type DON-producing parental strain. *Fg* was specifically used for this particular study because its facile transformation system and published genome sequence enabled the construction of the *Tri5* deletion strain. Inoculation of detached leaves with *Fg* Δ*Tri5* resulted in similar H_2O_2 production and cell death responses as for tissues inoculated with the wild-type *Fg* and *Fp* strains at early stages of infection (data not shown, Fig. 1; supplementary Fig. S1). Results also indicated that wheat defence gene transcript levels were unchanged between tissues inoculated with these two strains at 1, 2 and 14 days post-inoculation. However, a significant reduction in defence transcript levels (Fig. 5A) and fungal biomass (Fig. 5B) was observed in wheat stem tissue inoculated with the *Fg* Δ*Tri5* strain at 28 days post-inoculation.

DISCUSSION

Previous work has shown that production of DON plays an important part in fungal colonization of host tissues in both FHB

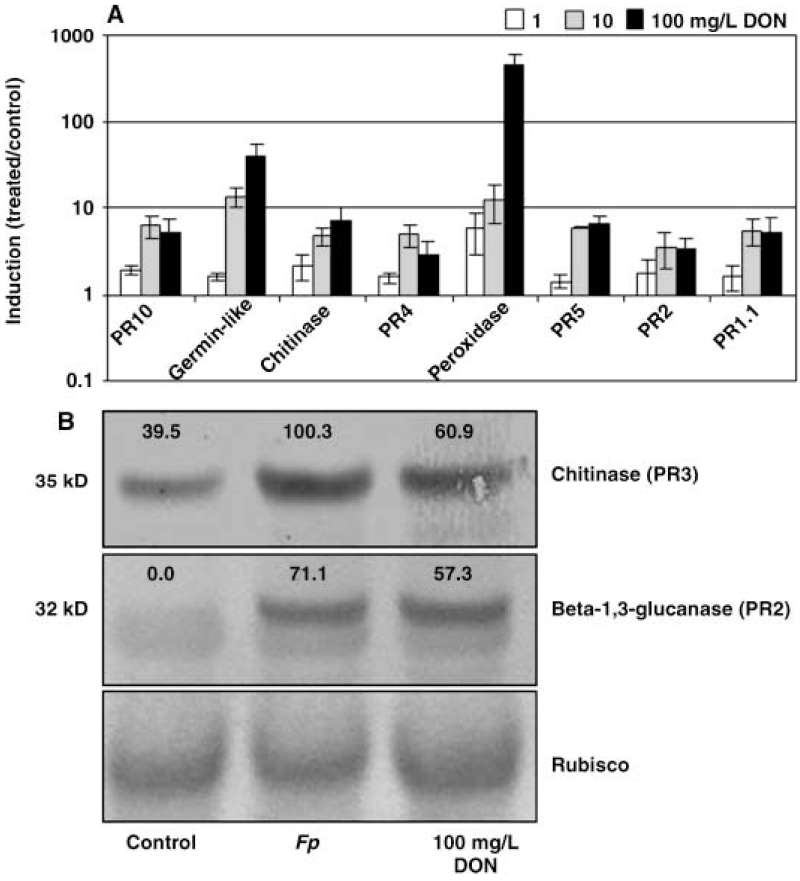

Fig. 3 (A) Induction of defence gene expression in 2-week-old seedlings at 1 day after treatment with 1, 10 and 100 mg/L DON. Columns represent average induction ratios (± SE; $n = 3$) of gene transcripts in treated compared with mock-treated and are plotted on a logarithmic scale. (B) Western blot analysis of total protein extracted from wheat tissue using β-1,3-glucanase (PR2) and chitinase (PR3) antibodies 2 days after mock treatment, *F. pseudograminearum* inoculation, or 100 mg/L DON treatment. Lower panel shows Rubisco stained with Ponceau red to show protein loading. The numbers above bands for chitinase and β-1,3-glucanase indicate protein levels as a percentage of Rubisco. Total protein (20 μg) was separated on a 4–12% polyacrylamide gradient gel. Protein molecular masses are indicated on the left.

and CR caused by the fungal pathogens *Fg* and *Fp* (Jansen *et al.*, 2005; Langevin *et al.*, 2004; Maier *et al.*, 2006; Mudge *et al.*, 2006). Interestingly, the work described here indicates that DON can also induce a range of classical plant defence responses in wheat, including the production of ROS that may be at least partially responsible for the induction of host defence gene transcripts, their protein products and PCD. This suggests that DON may assist necrotrophic growth of the pathogen by promoting host cell death, while also stimulating an antimicrobial defence response in the host. These contrasting effects of DON may influence the rate and extent of disease development during FHB and CR diseases of wheat.

Previous characterization of responses induced in Arabidopsis following exposure to a range of trichothecenes, including T-2 toxin, HT-2 toxin, diacetoxyscirpenol and DON, has shown that all of these toxins can cause cell death and the induction of defence gene transcripts (Masuda *et al.*, 2007; Nishiuchi *et al.*, 2006). In addition, it was demonstrated that protein synthesis, assessed by measuring the incorporation of [^{3}H]-leucine into proteins of an Arabidopsis cell suspension, was 50% inhibited by 1.5 mg/L DON. Although the accumulation of defence proteins was not studied in Arabidopsis, it was suggested that DON may be acting as a protein synthesis inhibitor at concentrations below the threshold required to activate Arabidopsis defence responses (Nishiuchi *et al.*, 2006). Consistent with these studies, our work showed that, in wheat, a concentration-dependent induction of defence gene transcripts occurred, with larger effects on defence gene induction, including defence protein accumulation, at higher concentrations (100 mg/L), which are known to inhibit protein synthesis in wheat (Miller and Ewen, 1997; Rocha *et al.*, 2005). In our experiments, we applied DON droplets to the surface of wheat tissue and it would have diffused into surrounding tissue. Thus, defence gene transcript and protein induction may have occurred in plant cells prior to DON reaching levels that inhibit protein synthesis. In affected cells, it is also possible that total protein synthesis may be reduced while the expression of defence genes we assessed was preferentially induced. However, this possibility appears to be unlikely in this situation as there was no reduction in Rubisco levels in DON-treated leaves. It is not clear from current studies if it is specifically DON that is detected

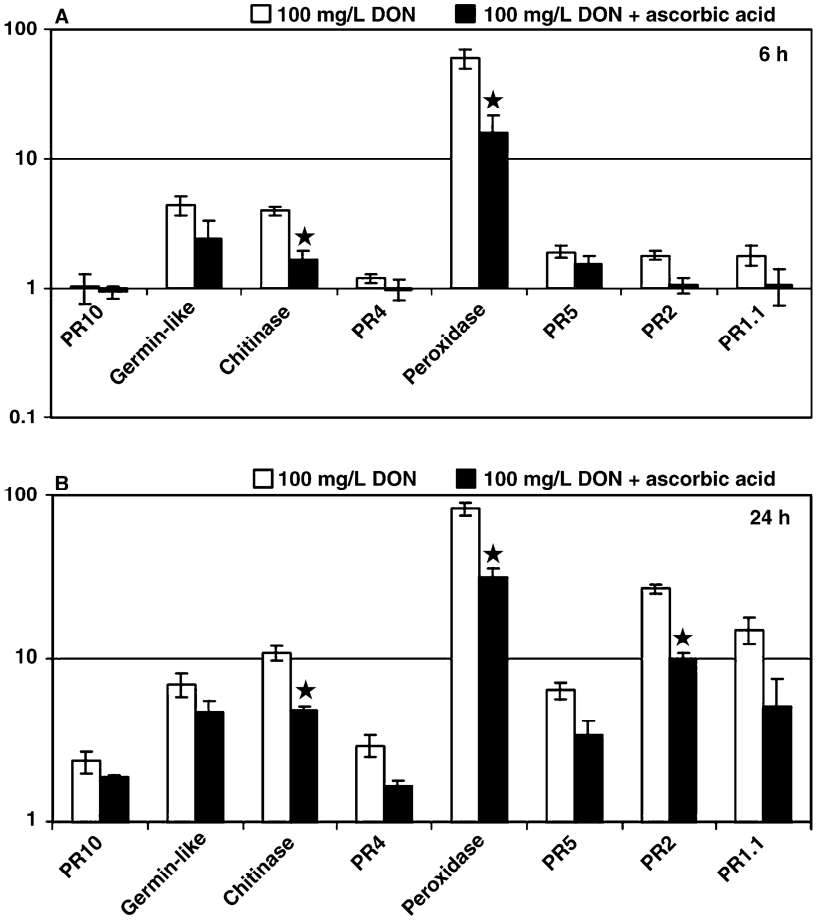

Fig. 4 Induction of defence gene expression in 2-week-old wheat seedlings (A) 6 h and (B) 24 h after treatment with 100 mg/L DON and a combination of 100 mg/L DON and 7 g/L ascorbic acid. Columns represent average induction ratios (± SE; $n = 3$) of gene transcripts in treated compared with mock-treated plants and are plotted on a logarithmic scale. Statistically significant differences in gene induction (Student's *t*-test, $P < 0.05$) resulting from treatments including ascorbic acid are indicated by a star.

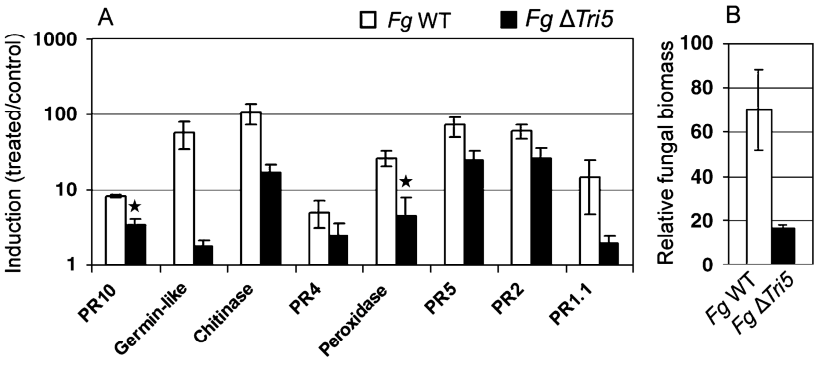

Fig. 5 (A) Induction of defence gene expression 28 days after inoculation with the DON-non-producing *F. graminearum Tri5* deletion line (*Fg* Δ*Tri5*) and wild-type. Columns represent average induction ratios (± SE; $n = 3$) of gene transcripts in treated compared with mock-treated plants and are plotted on a logarithmic scale. Statistically significant differences in gene induction (Student's *t*-test, $P < 0.05$) resulting from treatments are indicated by a star. (B) Fungal biomass 28 days after inoculation with the DON-non-producing *F. graminearum Tri5* deletion line and wild-type. Columns represent average amplification of the *Fg* 18S rRNA gene relative to wheat *actin* (± SE; $n = 3$).

by host cells leading to the induction of defence transcripts or if non-specific cellular stress caused by this toxin leads to the release of endogenous signals that activate defence responses in surrounding cells.

The most highly transcriptionally induced genes following DON treatment were the *germin-like* and the *peroxidase* genes, both of which are related to reactive oxygen metabolism (Liu *et al.*, 2005; Zimmermann *et al.*, 2006). These functional associations are consistent with the production of H_2O_2 that was also observed in wheat cells following DON infiltration. Application of the antioxidant ascorbate, together with DON, significantly reduced the level of transcriptional activation of the *peroxidase*

gene by DON, as well as that of several other defence genes. The effects of ascorbic acid on the activity of DON are unknown, but it is likely that reactive oxygen production in response to DON is at least partially responsible for the observed induction of host defence genes.

We have demonstrated here that DON causes cell death in wheat and that this was associated with genomic DNA laddering, a hallmark of PCD in plants and other eukaryotes (Ryerson and Heath, 1996; Tada *et al.*, 2001). PCD is known to be an active process that requires *de novo* protein synthesis (Tada *et al.*, 2001), and treatment with DON combined with cycloheximide, a eukaryotic protein synthesis inhibitor, prevented cell death 24 h after treatment. The mechanisms involved in this protective effect of cycloheximide are unknown, although it is possible that synthesis of a specific protein associated with DON-inducible cell death is inhibited by cycloheximide. If this is the case, it suggests that the cell death observed here is actively undertaken by host cells and is likely to be PCD rather than necrosis.

H_2O_2 is known to induce PCD in plant cells, (Houot *et al.*, 2001; Yoda *et al.*, 2006) and as expected, we observed that co-infiltration of DON and ascorbic acid reduced genomic DNA laddering and cell death, further suggesting that DON elicitation of ROS may be an important signalling event that stimulates PCD. Necrotrophic fungal pathogens often produce toxins to induce PCD during infection (Navarre and Wolpert, 1999; Tada *et al.*, 2001). The toxin fumonisin, produced by pathogens such as *F. verticillioides* and *F. proliferatum* (Munkvold *et al.*, 1999), has been demonstrated to induce cell death by depletion of extracellular ATP (Chivasa *et al.*, 2005). Stimulation of cell death by mycotoxins would release nutrients, facilitating necrotrophic fungal growth as well as spread throughout the host. This functional characteristic of DON is consistent with the reduced ability of DON-non-producing mutant *Fusarium* strains to infect spikelets surrounding the point of infection during FHB (Jansen *et al.*, 2005).

Host cell death and H2O2 production all appeared to require the application of relatively high concentrations of DON. Measurements of DON in inoculated crown and head tissues of wheat following inoculation with F. graminearum have shown that DON can accumulate to physiological levels on a fresh weight basis in excess of 100 μg/g (Mudge et al., 2006). However, these high concentrations of DON in infected tissues occur at relatively late stages after stem inoculation (14–28 days) and it is therefore likely that host responses to DON such as PCD are associated with the later stages of CR disease when visible lesions develop. We were able to investigate directly whether DON has a role in inducing wheat defence responses during CR disease development using a Tri5 deletion mutant of Fg. Defence gene induction was not significantly different between the two strains within the first 14 days post-inoculation and it was only at 28 days post-inoculation that induction of several defence genes was lower in tissue inoculated with the Fg ΔTri5 compared with wild type. This is consistent with previous observations where infection on wheat stems by the wild-type Fg resulted in DON levels of approximately 300 μg/g fresh weight at 28 days post-inoculation (Mudge et al., 2006). According to the results presented herein, these levels should be capable of inducing plant defence genes. However, it was also at this time point that significantly less fungal biomass was observed for the Fg ΔTri5 strain compared with the wild-type, and so it is unclear if reduced fungus or a lack of DON is influencing defence gene expression at this later stage of infection. Recently, Ansari *et al.* (2007) also reported that a small suite of genes in wheat heads were inducible by DON, but there was no difference in the expression of these genes in wheat heads during the early stages post-inoculation with a DON-non-producing mutant of Fg and a wild-type strain (Ansari *et al.*, 2007). So even though we saw several parallels between wheat responses induced by DON and those induced following inoculation with DON-producing Fp and Fg, it is likely that these fungal pathogens may produce many other elicitors of host responses, and that DON is not likely to be a primary determinant of the host defence responses during early stages of fungal infection.

During later stages of disease, DON production may elicit defences such as the accumulation of the chitinase and glucanase proteins studied here. It may be that the effectiveness of these defence proteins is one of the factors determining fungal colonization rates and therefore quantitative resistance responses. A recent study has indeed suggested that transgenic wheat plants expressing *PR2* (β-*1–3 glucanase*) showed reduced FHB development (Mackintosh *et al.*, 2007). Our experiments suggest that the level of production of DON affects wheat cellular responses in a way that could either promote necrotrophic fungal growth by initiating PCD or reduce fungal growth by triggering defence gene expression and accumulation of antimicrobial proteins.

EXPERIMENTAL PROCEDURES

F. pseudograminearum isolate, plant material and inoculation procedure

Spore suspensions of the aggressive *Fp* isolate CS3069 from the CSIRO collection were cultured as previously described (Desmond *et al.*, 2005). The commercial variety of hexaploid wheat (*Triticum aestivum*) cv. Kennedy was grown in glasshouse conditions as previously described (Desmond *et al.*, 2005). Inoculations were performed using a detached leaf assay where leaves of 2-week-old plants were cut into segments ~4 cm long and placed on moist tissue in a Petri dish and then 10-μL drops of spore solution were evenly spaced along the leaves ~0.5 cm apart. Samples were collected ~7 days later for DAB and trypan blue staining.

Infiltration of wheat tissue for DAB and trypan blue staining

Wheat leaf segments from 2-week-old plants were infiltrated with the following solutions: (1) 100 mg/L DON, (2) 200 mg/L DON, (3) 100 mg/L DON and 7 g/L ascorbic acid, (4) 100 mg/L DON and 4 mg/L cycloheximide, and (5) distilled water. Infiltration was performed by covering tissue with treatment solutions and applying a vacuum for 15–20 min. Stem segments were then left in solution for ~10 min before being removed and placed on moist filter paper in a Petri dish. After infiltration, samples were collected 6 and 24 h later and stained using DAB to reveal H_2O_2 production and trypan blue to reveal cell death. This experiment was performed twice, with similar results produced on both occasions. Images presented are representative of the results.

DAB and trypan blue staining of inoculated and infiltrated tissue

DAB staining to reveal H_2O_2 was carried out using the DAB Liquid Substrate System (Sigma, St Louis, MO) according to the manufacturer's directions. Tissue was de-stained by boiling tissue in 90% EtOH for 1 min and viewed using a light microscope. Trypan blue staining was performed to reveal dead plant cells using the procedure described by Belenghi *et al.* (2003) with a further dilution of the stock solution described with two volumes of 96% ethanol. Tissue was immersed and boiled in the trypan blue solution for 1 min and left to stain overnight before de-staining with chloral hydrate and viewing using a Leica MZ16FA stereo light microscope.

Infiltration of wheat tissue for DNA cleavage analysis

Wheat stem segments ~2 cm long, collected from 2-week-old Kennedy plants, were infiltrated using the same procedure described above with treatments of 100 mg/L DON and a combination of 100 mg/L DON and 7 mg/L ascorbic acid. DNA degradation was assessed by extracting genomic DNA 24 h after infiltration as previously described (Kazan *et al.*, 1993), followed by electrophoresis through a 1% agarose gel made with TAE buffer and stained with ethidium bromide. Digital analysis of the gel images was performed using ImageJ software (http://rsb.info.nih.gov/ij/). First, the image was converted to 8-bit binary and the threshold was set from 0 to 160. Total pixels was estimated for each lane using the Analyse Particles function with default settings and this was compared with the number of pixels in each lane that were below the 5000-bp band from the 1-kb + DNA ladder (Invitrogen, Carlsbad, CA, USA). This method of treatment was found to provide the most consistent results, most likely because infiltration allows even treatment of all cells used for analysis. DNA laddering was observed in samples prepared on three separate occasions, and the images presented are representative of the results observed.

Drop treatment with DON and ascorbic acid for gene expression analysis

Deoxynivalenol (Sigma) was initially dissolved to 10 g/L in 100% methanol and then further diluted with water to final concentrations of 100, 10 and 1 mg/L. For treatments of DON combined with an antioxidant, a solution of 100 mg/L DON plus 7 g/L ascorbic acid (pH ~7) was used. All treatment solutions also contained 0.05% Tween 20. Wheat plants were treated by placing a 10-μL drop onto the stem base using the same method previously described for *Fp* inoculation (Desmond *et al.*, 2005).

Gene expression analysis by reverse transcriptase quantitative PCR

Tissue samples were collected for three independent biological replicates 1 day after treatment with DON or DON combined with ascorbic acid, followed by RNA isolation and cDNA synthesis as previously described (Desmond *et al.*, 2005). Real-time quantitative PCR conditions, including primer details and analysis of results, have been previously described (Desmond *et al.*, 2005).

Defence protein expression analysis by protein extraction and Western blot

Total protein was extracted from ~200 mg of frozen wheat stem base tissue from 2-week-old seedlings as described (Jayaraj *et al.*, 2004), with the following alterations: one tablet of complete protease inhibitor (Roche, Mannheim, Germany) was added freshly to 50 mL of extraction buffer instead of PMSF. After tissue homogenization in the extraction buffer, samples were left on ice for 15 min. Protein concentration was determined using the bicinchoninic acid microtitre plate assay kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Proteins (75 μg) were separated using a Nu-Page 4–12% bis-tris pre-cast gel (Invitrogen) electrophoresed in a Novex Mini-Cell (Invitrogen) and transferred to a nitrocellulose membrane using an XCell II Blot module (Invitrogen) followed by protein detection, all performed according to the manufacturer's instructions. For size determination, the SeeBlue Plus2 (Invitrogen) pre-stained standard was included in all gels. Primary antibodies were kindly donated by S. Muthukrishnan, Kansas State University. Secondary antibodies were detected by incubating blots in one-step NBT/BCIP (Pierce) for 5–10 min before stopping the reaction with water. To quantify protein levels, ImageJ was again used to count pixels for each band and levels relative to Rubisco were determined. ImageJ settings included a threshold of 10–116 and the Analyse Particle function was run with default settings.

Generation of *Fg Tri5* deletion lines

Fusarium graminearum isolate CS3005 (Akinsanmi *et al.*, 2006) was used as a parent to construct the *Fg* Δ*Tri5* strain. The 2.8 kb of the *Tri5* genomic locus was amplified (AATCTATCAGTGCTTAAATGCAGTTCC and TACGTAGGCCGCGCAGAGGTCAGTA), cloned into pCR8/GW-TOPO (Invitrogen) and the *Hin*dIII–*Nco*I fragment of the *Tri5* coding sequence replaced with the hygromycin phosphotransferase cassette from pUChph, leaving two flanks of 1.2 kb. The construct was introduced into CS3005 protoplasts generated from approximately 1×10^7 conidia germinated overnight in 200 mL of TB3 media (0.3% yeast extract, 0.3% Casamino acids, 20% sucrose) at 20 °C with shaking at 150 r.p.m. Germlings were collected on miracloth and washed with 1 M sorbitol before being resuspended in 20 mL of 1 M sorbitol containing 5 U chitinase (Sigma-Aldrich, St Louis, MO), 500 mg Driselase (Sigma-Aldrich) and 200 mg lysing enzymes (Sigma-Aldrich), and incubated for 1–2 h at 28 °C to release protoplasts. Protoplasts were collected by centrifugation at 2600*g*, 4 °C, washed three times with ice-cold STC (20% sucrose, 50 mM Tris/HCl, pH 8, 50 mM $CaCl_2$). To a 200-μL aliquot of STC containing 2×10^7 protoplasts, 10 μg circular plasmid DNA was added, incubated for 20 min at room temperature, then 1 mL of 40% PEG_{4000}, 10 mM Tris HCl, pH 8, and 50 mM $CaCl_2$ was added, incubated for 20 min at room temperature and then 5 mL of TB3 media was added and the tubes incubated overnight at room temperature with gentle agitation. Three millilitres of the transformation mixture was embedded in 30 mL regeneration media (0.2% yeast extract, 0.2% Casamino acids, 0.55% low-melt agarose, 27% sucrose) and set in 14-cm Petri dishes, incubated overnight and then overlaid with 30 mL of regeneration media containing hygromycin (Roche, Penzberg, Germany) at 400 mg/L. After 5–7 days of growth at room temperature in the dark, transformants were transferred to individual plates containing hygromycin (200 mg/L), allowed to grow, single spored then checked for insertion of the construct by PCR (data not shown).

Inoculation of wheat with *Fg*

Wheat (cv. Kennedy) was grown as described above. Seedlings were inoculated with the wild-type *F. graminearum* isolate CS3005 and the *Fg* Δ*Tri5* strain in parallel at the stem base as described (Mitter *et al.*, 2006).

Estimation of fungal biomass in wheat stems inoculated with *Fg* Δ*Tri5* and parent *Fg*

Samples were collected 1, 2, 14 and 28 days post-inoculation by collecting stem from the crown to the first leaf. Each time point consisted of three biological replicates taken in parallel and each replicate was a pool of eight stem bases. DNA was extracted from the samples using a QIAGEN Dneasy® Plant Mini Kit according to the manufacturer's instructions. Real-time quantitative PCR was performed as described above using the *Fg* 18S rRNA gene and wheat *actin* primers (Mudge *et al.*, 2006). Internal PCR amplification efficiencies were calculated by using the program LinRegPCR 7.5 (Ramakers *et al.*, 2003) and absolute DNA amplification was calculated by the average efficiencies raised to the negative crossing threshold. *Fg* biomass was estimated as the absolute amplification of fungal DNA relative to the absolute amplification of wheat DNA.

Analysis of gene expression in *Fg* inoculated tissue

RNA was extracted from the same samples used for estimating fungal biomass followed by cDNA synthesis as described above. RT-qPCR was performed to assess defence gene expression using the primers, procedures and analysis described above.

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SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article:

Fig. S1 H_2O_2 production, detected using DAB stain, in wheat leaf tissue 6 h after infiltration with (A) mock, (B) 100 mg/L DON, (C) 100 mg/L DON and 7 mg/L ascorbic acid, and (D) 100 mg/L DON and 4 mg/L cycloheximide. Cell death, detected using trypan blue stain, in wheat leaf tissue 24 h after infiltration with (E) mock, (F) 100 mg/L DON, (G) 100 mg/L DON and 7 mg/L ascorbic acid, and (H) 100 mg/L DON and 4 mg/L cycloheximide.

Fig. S2 Pathogen-induced cell death stained using trypan blue 7 days after inoculation. Infection follows vascular tissue and is most widespread in stomatal guard cells.

This material is available as part of the online article from:
http://www.blackwell-synergy.com/doi/full/10.1111/j.1364-3703.2008.00475.x
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Chapter 7: Conclusions and Future Directions

Fusarium crown rot (FCR) and head blight (FHB) of wheat are diseases of major economic importance because they cause large amounts of yield loss and toxin contamination in food products. The host-pathogen interaction between *F. graminearum* and wheat is a complex one where host resistance is not a gene-for-gene model as in other pathosystems. A review of the current literature has shown that the majority of functional genomics research involving *Fusarium* incited wheat disease has focused primarily on FHB and little work has involved FCR. Crown rot is a *Fusarium* infection of the wheat stem base, but until now, neither the specific colonisation pathway nor the molecular interactions with the plant host had been described. Importantly, there has also been no prior assessment of whether virulence genes exist in the pathogen that are specialised for particular host tissues and the FCR and FHB disease types.

CHARACTERISATION OF THE *F. GRAMINEARUM* COLONISATION PATHWAY DURING FCR OF WHEAT

An interesting observation made at the early stages of this study was that the fungal biomass fluctuates substantially during the time course of disease development for FCR. Of particular note was the period of 2-14 dpi, termed phase 2 of infection where fungal biomass ceased to increase and declined. During this phase of FCR infection, it therefore appears that the wheat plant presents the fungus with significant challenges that result in a dramatic decrease in fungal biomass. These challenges may be multi-dimensional in nature and when combined they prove successful in temporarily slowing down the infection and this may be the underlying reason why FCR disease develops over a much longer time frame that FHB. It is known that a diverse array of host defence response genes are activated in the stem base within 1-2 days of inoculation of wheat stem bases with *F. pseudograminearum* (Desmond *et al*., 2005, Desmond *et al*., 2008b, Desmond *et al*., 2008a) and similar defence responses were observed following inoculation with *F. graminearum* (Desmond *et al*., 2008b)(Chapter 6). From the host Affymetrix array experiments of (Desmond et al., 2008a) it appears that wheat defence responses include the production of anti-fungal proteins, enzymes for the production of potential phytoalexin-like compounds and structural barriers. Chemical based defence responses from the host present a major challenge to the fungus, this probably includes an oxidative burst and these responses most probably induces the transcription of *F. graminearum* detoxification associated genes such as superoxide dismutase. It is possible that slowing down of fungal colonisation at phase 2 may be time for the fungus to adapt to, and

overcome antifungal molecules produced by the host. Physical barriers within the plant structure also challenged the fungus in several stages throughout the infection. However, during phase 2 the fungal infection was confined to the outer leaf sheath and the fungus appeared either to be unable to colonise horizontally into the centre of the stem or was able to follow a more amenable pathway for colonisation vertically downwards towards the crown through the interior of the outer leaf sheath. This appears to occur without a large increase in fungal biomass and it may be that the fungus uses a stealth strategy at this stage where it minimises its impact on the host and in turn minimises the host response it has to deal with. Further analysis of this stage of the infection process at the cellular level is warranted.

As discussed in Chapter 2, the wheat seedling stem is not solid but composed of emerging leaf sheaths. The challenge that the fungus encounters through the physical barriers in the plant structure is to a large extent associated with, and changes with, the physical development of the plant. For example, once the fungus has colonised the plant crown tissue it does not vertically colonise the stem parenchyma tissue toward the wheat head in phase 3 until the plant has matured and stem elongation has occurred (Mudge *et al*., 2006). This also suggests that there is a need for the fungus to evolve and express colonisation factors such as DON at the later stages of infection to facilitate more systemic invasion of the host. It is interesting that DON appears to have to reach quite high concentrations (>50 ppm) to effect wheat cell death (Desmond *et al*., 2008a) and this may take time and the substantial build up of fungal biomass before these concentrations are reached.

FUNCTIONAL CHARACTERISATION OF TWO *F. GRAMINEARUM* GENES DURING FCR AND FHB

One of the objectives of this project was to determine whether there were any fungal genes that played specific roles in virulence for FCR and FHB diseases. During this project two new *F. graminearum* virulence genes (*FgABC1* and *FgSOD1*) were identified from genes that were differentially regulated in the fungus during FCR disease development. The ABC transporter gene *FgABC1* identified in this project is the first *F. graminearum* virulence factor to be described with a role that appears to be specific for crown rot disease of wheat. Interestingly, although deletion of this gene appeared to reduce FCR disease symptoms there was no statistically significant effect on fungal biomass production in the host. It is possible that this compound may be a fungal derived effecter that plays a role in triggering the visual necrotic lesion development characteristic to

Fusarium crown rot but fungal development is not affected. It is therefore debatable as to whether this gene is a true virulence gene but in practical terms any gene that has an impact on the development of symptoms has a role in disease development and its impact on yield. Ultimately, in respect to developing a disease control it is possible that FgABC1 function could be blocked using an introduced compound. This compound could be chemically synthesised and applied as part of a crop treatment, or expressed by a genetically enhanced wheat plant. It is therefore considered important to determine the identity of the active compound transported by FgABC1.

During both FCR and FHB superoxide dismutase *FgSOD1* shows similar transcription profiles but it was only significantly associated with the virulence of *F. graminearum* during FHB. It is hypothesized (Chapter 4) that this may be due to a larger oxidative burst manifesting in the wheat head therefore creating a greater need for the SOD detoxification enzyme in this tissue. If this were the case, then it would be important to study and compare the amount of reactive oxygen species released by the wheat host in its different organs specifically the wheat head and the crown. It would also be valuable to determine whether different cultivars of wheat exhibit variation in the oxidative response to *F. graminearum* during FCR and FHB and if this variation can be linked to increased resistance to *Fusarium*-incited diseases. It was also found that *FgSOD1* showed variation in its transcription profile based on assays using independent probe sets and thus suggests that this gene may undergo alternative mRNA splicing. If this was the case it may change our current understanding of the host-pathogen interaction in regards to oxidative burst and oxidative stress responses. Questions to be addressed would include; are there different protein products encoded by *FgSOD1*? If so what are they and what are their functions?

MOLECULAR COMPARISON BETWEEN FCR AND FHB

Investigating and understanding the host-pathogen interaction between *F. graminearum* and wheat is important because mechanisms of pathogenicity and defence can be identified as targets for disease control. A comparative bioinformatic analysis of FCR microarray data and previously published FHB microarray data (Guldener *et al*., 2006b) suggested that the global gene expression profiles of *F. graminearum* were significantly similar between FCR and FHB in the early stages of infection but the fungal gene expression patterns in the two diseases became more diverse as both infections progressed. This type of comparative functional analysis of large datasets demonstrates the power of robust multiple array (RMA) normalisation, the use if standard gene expression analysis platforms like Affymetrix system, combined with the growing amount of large functional

datasets publically available on website-databases. These comparisons are based on analysis of the whole transcriptome and although they are suggestive of common and distinct functions in infection at early and late stages of infection they do not reflect the role of individual fungal virulence genes in these two diseases. This would require functional analysis on a gene by gene basis.

In light of this information, seven *F. graminearum* virulence and pathogenicity genes known to play an important role in FHB were tested to identify if they also played a role during FCR. The results described in this thesis showed that some pathogenicity and virulence systems are common to both FCR and FHB, but amongst this selected set there were also genes and associated mechanisms that seemed to act specifically for FHB. This information is important when targeting genes for disease control of *F. graminearum* in the field. If a control strategy was being developed to interfere with one of the known virulence or pathogenicity genes of *F. graminearum*, it would hold greater value to target a gene that was important for FHB and FCR rather than one that acts specifically in a particular disease. The work, together with that on *FgABC1*, also highlighted the fact that there can be a difference between the role a gene plays in inciting visible symptoms and the ability to facilitate colonisation. The *Tri5* gene discussed in Chapter 6 was identified as a colonisation factor for both FCR and FHB, therefore when conducting host-pathogen interaction studies in regards to disease severity as a whole, it is important to measure multiple aspects of the disease.

All of the genes identified as belonging to the *Fusarium* infection program have very similar expression profiles during both FCR on wheat and FHB on barley (Chapter 2). It is very interesting that these virulence and pathogenicity genes can have similar expression profiles and yet can be important functionally for one specific disease. It is possible that *F. graminearum* does not induce specific sets of pathogenicity genes as a result of recognising components of either wheat stem or head tissue. Instead this pathogen may induce all pathogenicity genes needed for any infection. If this is the case, identifying and interfering with the initial sensing systems that trigger the induction of the pathogenicity and virulence factors may prove a powerful technique in controlling all *F. graminearum* incited infections of wheat and barley. *Fusarium graminearum* has the ability to cause disease in many other small grain cereals and maize. Thus it would be interesting to know if all the pathogenicity genes that are induced for infection of wheat are also induced for infection of other susceptible hosts.

A large number of differentially regulated *F. graminearum* genes during FCR are of unknown function. As discussed in Chapter 2, many molecular, genetic and proteomic studies of other

fungal-plant interactions also identified genes and proteins that are yet to be functionally characterised. In the current post-genomics era where whole genomes are commonly sequenced it is now possible to systematically characterise these genes to unlock novel pathways and interactions that to date have eluded scrutiny. There is no doubt that many future projects will be dedicated to analysing these genes of unknown function. Because *F. graminearum* can be cultured in a haploid state in the laboratory and gene knock-out constructs can be created in a small series of PCR reactions using the split-marker system and transformation protocols are becoming more efficient, it is foreseeable that every gene of *F. graminearum* could be systematically knocked out for functional analysis. As the molecular side of this approach develops it will be necessary to develop bioassays for pathogenicity and virulence that have a higher throughput than the assays used for FCR and FHB in this study. Both of the current assays take months to perform and would not be well suited to whole genome scale functional analysis of virulence genes in *F. graminearum.* Thus, there is considerable scope for innovation in both the pathology and molecular biological approaches needed to completely dissect the virulence requirements of *F. graminearum* on wheat.

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APPENDIX 1:

Table A1: Gene specific primers used to create the 'knock-out' vector for fungal transformation and gene deletion.

FGSG_01831 F5'	5′- ATACGTGACAATGTTGATCG – 3′
FGSG_01831 R5'	5′- TCCTGTGTGAAATTGTTATCCGCTCACGAGTTTGTGACCTTGTA – 3′
FGSG_01831 F3'	5′- GTCGTGACTGGGAAAACCCTGGCGTTGGATGTGCTTTGAGGATGTG – 3′
FGSG_01831 R3'	5′- CGGTCAACGTTTACGATCCC – 3′
FGSG_04647 F5'	5′- GGTGGCACTGATAAAAATGT – 3′
FGSG_04647 R5'	5′- TCCTGTGTGAAATTGTTATCCGCTTGTGATTATTGTAGGTGTTG – 3′
FGSG_04647 F3'	5′- GTCGTGACTGGGAAAACCCTGGCGAAGCCAATCGTGAATGATACCC – 3′
FGSG_04647 R3'	5′- TTGATCAAATGGCCGAGGTC – 3′
11296-pan F	5′- TAAAAGGGATGGGAAGATTCTCAGGTCTCGAGATAATAGCGTCCTAGAAGAACTGGAGGGGTGGT – 3′
11296-pan R	5′- TCTGTGGTATCTTTCGGCATCGTTAGAGATGGGTCGTTGGGCGAGGCTTTAACCTGAGGCTATGG – 3′
11164-pan F	5′- GACGGGGCTGACGCCACCACCCTCAGAGGTGTCTCCCCTGTGCAGAGAAGAACTGGAGGGGTGGT – 3′
11164-pan R	5′- GCCAGCGCTGGTGAATTCCCCTTCATCGTCAGCATCACCAACAACGCTTTAACCTGAGGCTATGG – 3′

Constructs for genes FGSG_01831 and FGSG_04647 were generated using the split marker protocol as described in Chapter 3. Constructs for FGSG_11296 and FGSG_11164 were generated using a recombination technique with the same *Escherichia coli* DY380 strain and vectors pCR8-GW/TOPO and pAN9.1 as described in (Gardiner et al., 2009a).

Table A2: Primers used in the screen for FGSG_11164 and FGSG_11296 homologous recombination

Fg11164screen_F	5′-cacgagagacaacaggaacg-3′
Fg11164screen_R	5′-tcggaagaggccatgagata-3′
Fg11296screen_F	5′-gtaggcaggcgtagccatta-3′
Fg11296screen_R	5′-cgaacattccaaaggagcat-3′

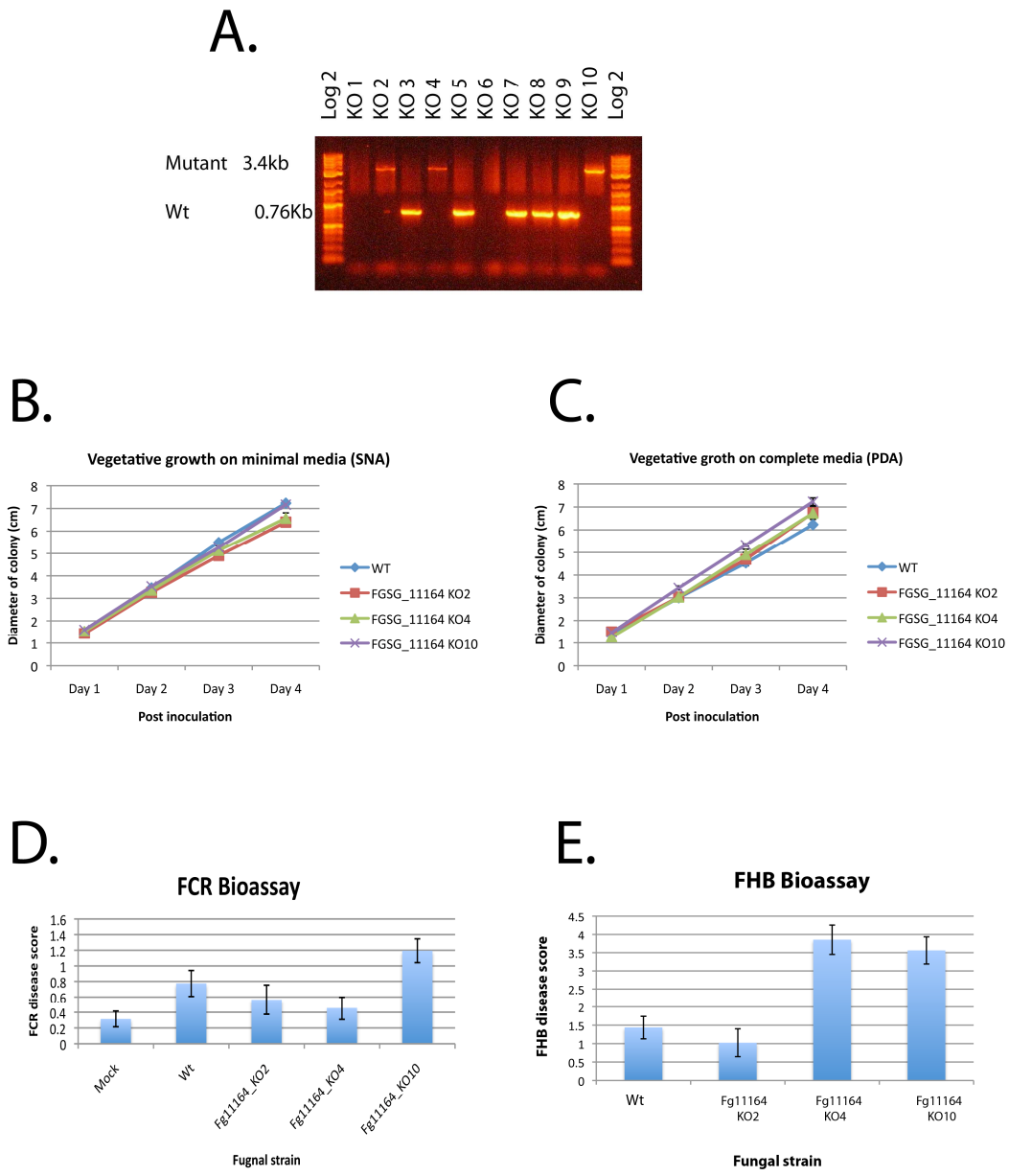

Figure A1: Screening of FGSG_11164 mutants and phenotypic analysis. PCR screen of putative mutant isolates where mutant band is 3.2kb and Wt band is 0.76kb (A). Vegetative growth of *F. graminearum* Wt and mutants on (B) defined minimal media and (C) complete media. All error bars are the standard error of the mean for three biological replicates. FCR disease severity of wheat infected with water (mock), wild type and the three independent FGSG_11164 mutants, each treatment contains 25 to 30 biological replicates (D). FHB disease severity of wheat heads infected with *F. graminearum* wild type and mutants (E), each treatment contains 13 to 15 biological replicates.

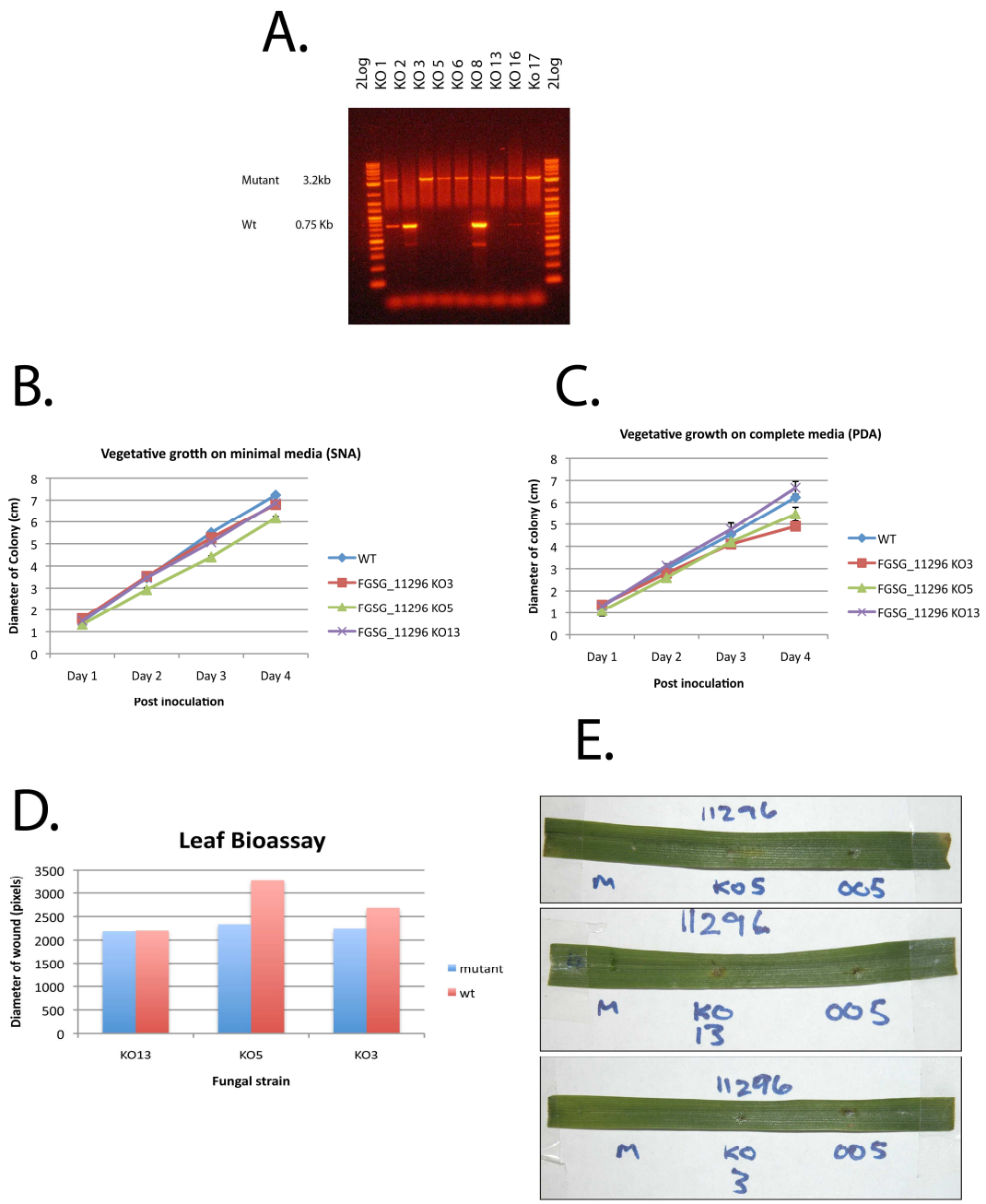

Figure A2: Screening of FGSG_11296 KO mutants and phenotypic analysis. PCR screen of putative mutant isolates where mutant band is 3.2kb and Wt band is 0.75kb (A). Vegetative growth of *F. graminearum* Wt and mutants on (B) defined minimal media and (C) complete media. All error bars are the standard error of the mean for three biological replicates. Wild type and mutant pathogenicity assay of wheat leaf (D) and representative images of assay (E).

APPENDIX 2:

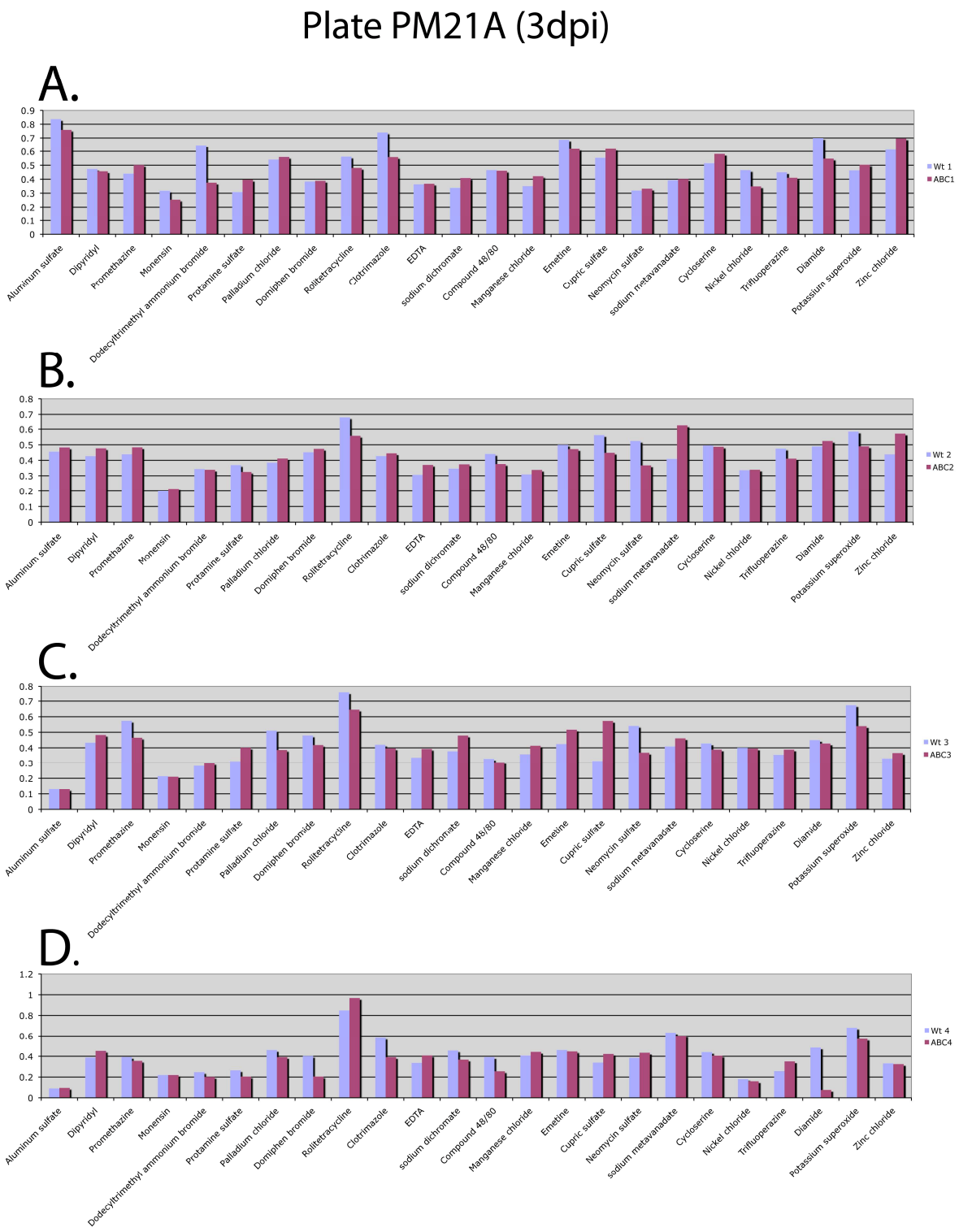

Figure A3: Average of the OD readings from two biological replicates for *FgABC1* mutant and Wt strains at 3 dpi at four different concentrations. Concentration (A) being the lowest and (D) being the highest for the respective compound.

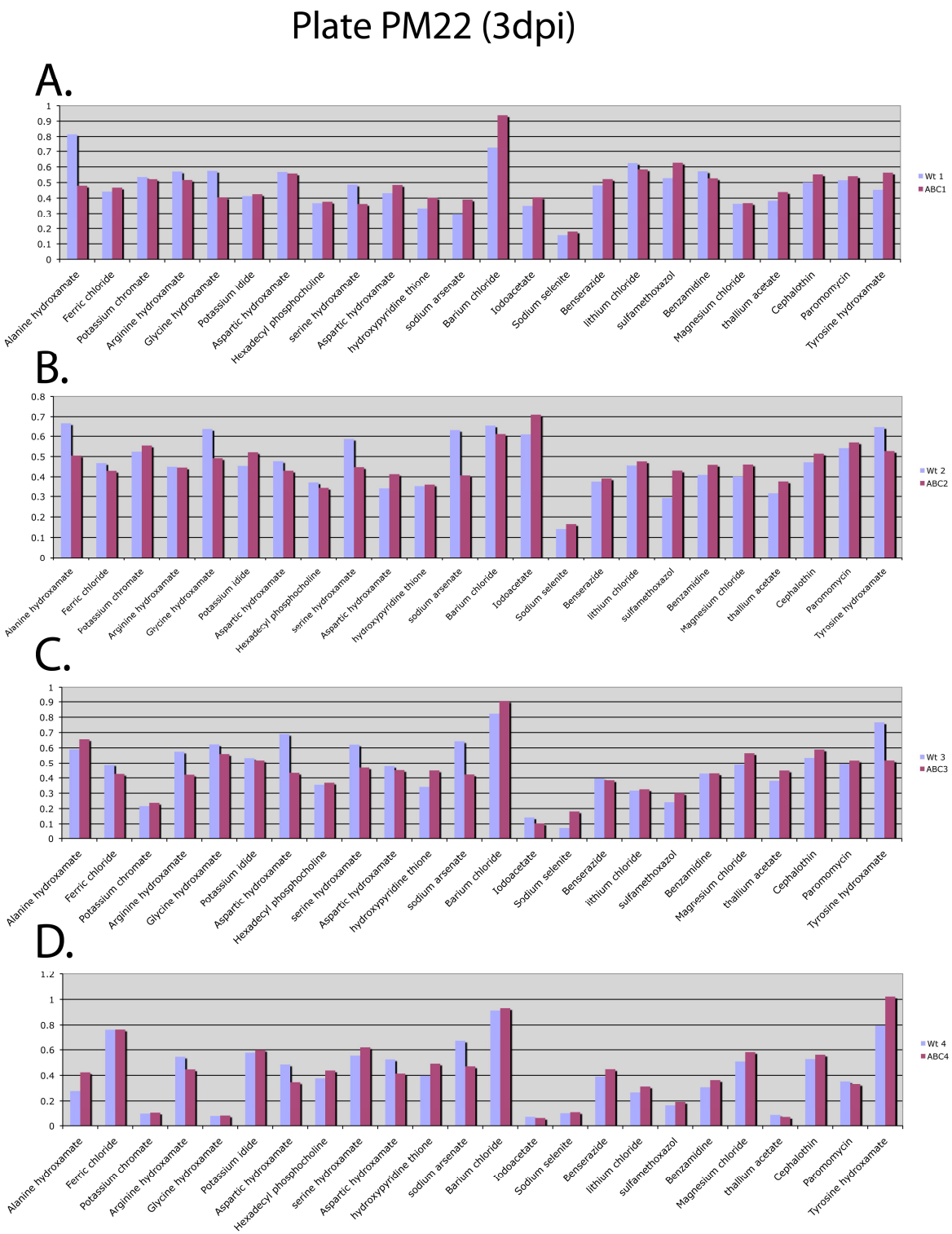

Figure A4: Average of the OD readings from two biological replicates for *FgABC1* mutant and Wt strains at 3 dpi at four different concentrations. Concentration (A) being the lowest and (D) being the highest for the respective compound.

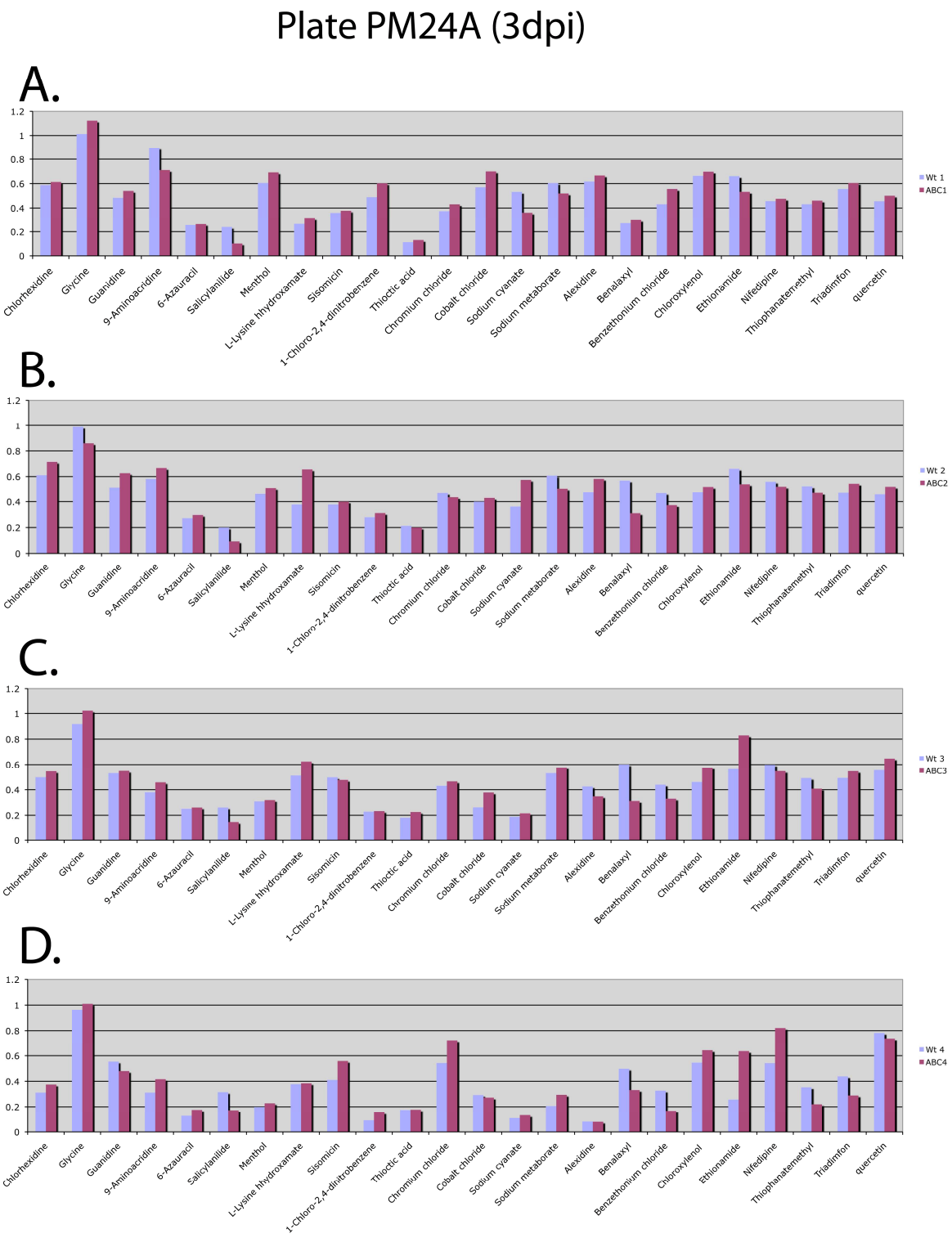

Figure 5A: Average of the OD readings from two biological replicates for *FgABC1* mutant and Wt strains at 3 dpi at four different concentrations. Concentration (A) being the lowest and (D) being the highest for the respective compound.

APPENDIX 3:

Results

BIOMASS OF Δ*TRI5FG* COMPARED TO WILD TYPE *F. GRAMINEARUM* DURING FCR OF WHEAT.

Results obtained at Rothamstead Research (Chapter 5) showed that at 28 dpi, visible symptoms induced by the *F. graminearum Tri5* deletion mutant were similar to the wild type, but the mutant accumulated significantly less fungal biomass indicating that the *F. graminearum Tri5* gene plays a role in the ability of the fungus to colonise the wheat plant during crown rot disease. At 28 dpi FCR is in transition between phase 2 and 3 of infection, as described in detail in Chapter 2. One difficulty in comparing the results obtained in Rothamsted in the UK and those obtained at CSIRO in Australia is that different isolates had to be used because of quarantine constraints and also different growth facilities. To investigate if DON had an effect on fungal biomass accumulation during other phases of FCR, wheat seedlings were infected with local Australian wild type CS3005 *F. graminearum* and a CS3005 *Tri5* deletion mutant Δ*Tri5Fg* (Desmond *et al*., 2008b) and samples were collected at 2, 14, 28 and 31 days post inoculation. Fungal biomass was estimated by RT-qPCR in the wild type and Δ*Tri5Fg* FCR time course and results show that in the early stages of FCR infection, 2 dpi and 14 dpi, the biomass of the mutant and wild type was not significantly different. However, at 28 dpi the wild type *F. graminearum* infection had accumulated on average ~4.24 fold more biomass than the Δ*Tri5Fg* infection confirming that the results obtained at Rothamsted are part of system of infection common to varying wheat varieties and *F. graminearum* isolates. However, the advantage in colonisation that DON gives the wild type over the mutant was short lived, with Δ*Tri5Fg* increasing in biomass to near wild type levels at 31 dpi (Figure). This indicates that DON produced by *F. graminearum* positively enables the fungus to colonise the infected host in the late stages of phase 2 during FCR infection but does not affect overall colonisation at later stages of infection.

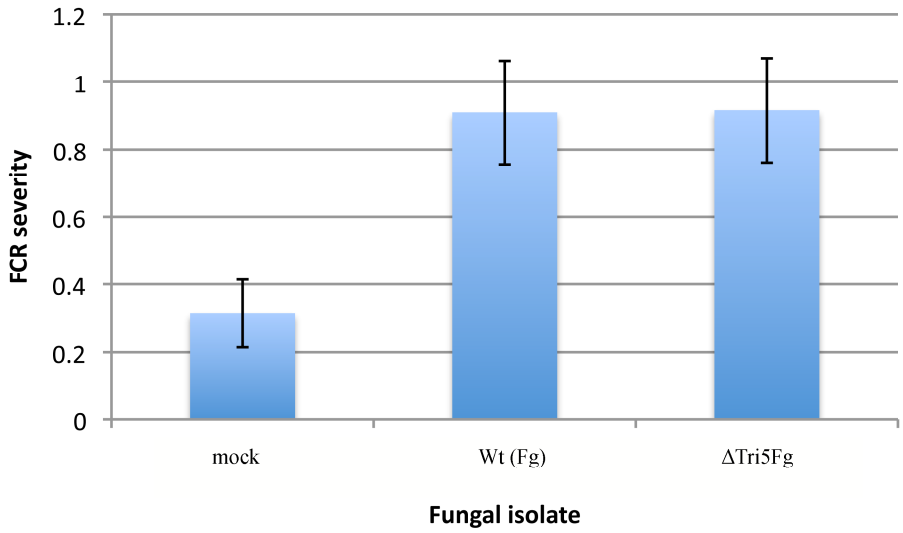

Figure A6: FCR severity of wheat seedlings at 31 dpi as a result of FCR infection caused by Δ*Tri5Fg* and wild type. All error bars are the standard error of the mean for 30 independent biological replicates.

TRI5 GENE EXPRESSION DURING FCR OF WHEAT.

Thus far results suggest that DON production plays a role in colonisation during the transition between phase 2 and 3 (28 dpi) of FCR but not during later stages of phase 3 (31 and 35 dpi). To understand why DON may be acting as a colonisation factor only at this time point, it was important to identify when the production of DON was initiated by determining when the *Tri5* gene was expressed and if that correlated to the rapid colonisation we see at 28 dpi. To do this, quantitative real-time (qPCR) amplification of RNA sequence for *F. graminearum 18S rRNA* and *Tri5* was performed on the samples in the large time course to determine the level of transcription of the *Tri5* gene at the time points sampled (Figure). In this experiment the *Tri5* gene maintained a similar level of expression over the course of infection from 2 dpi to 31 dpi with considerable variability at 14 dpi although this was not a statistically significant change in gene expression at this time-point. It is noted that in Chapter 2 it was shown that *Tri5* gene had much higher levels of expression *in planta* compared to axenically cultured vegetative mycelia. Thus, the trichothecene biosynthesis pathway is triggered in the very early stages of the plant-pathogen interaction and does not appear to change significantly throughout the phases of infection. A similar expression profile for *Tri5* was observed during FCR in the Affymetrix experiment and this was highlighted in Chapter 3.

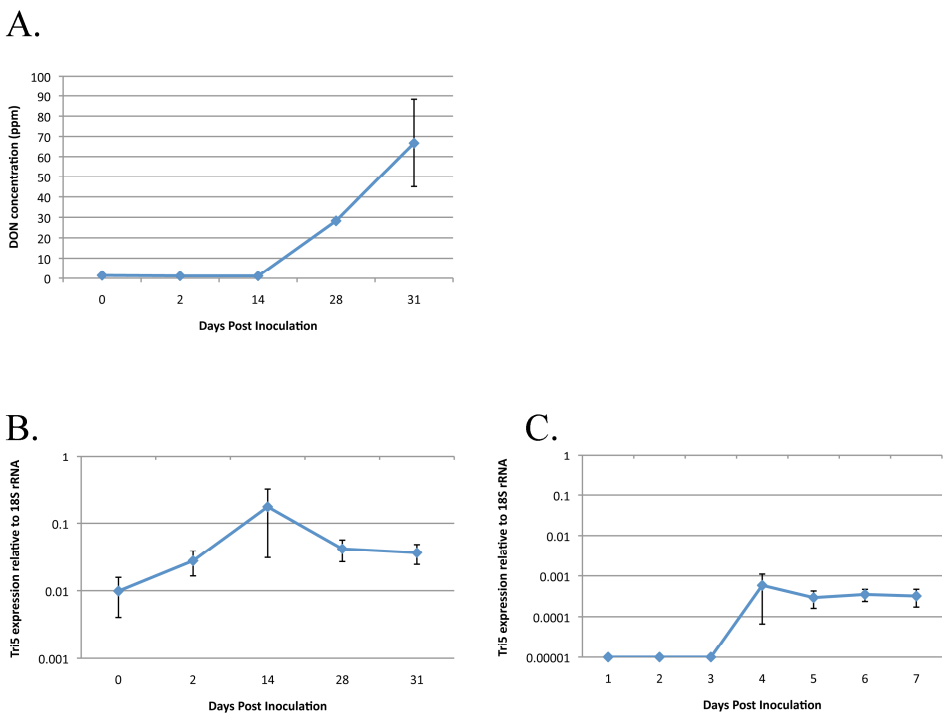

Figure A7: Analysis of DON myctoxin production by *F. graminearum* during both FCR and FHB of wheat. (A) DON concentration during FCR measured as ppm. (B) Relative *Tri5* transcription during FCR (C) Relative *Tri5* transcription during FHB. All error bars are the standard error of the mean for biological replicates.

QUANTITY OF DON ACCUMULATED DURING CROWN ROT REFLECTS BIOMASS ACCUMULATION AND *TRI5* GENE EXPRESSION.

It was shown that *Tri5* expression is relatively level throughout infection phases 2 and 3, therefore the next step was to determine the concentration of DON in the same samples and this was achieved by using an ELISA assay Deoxynivalenol (DON) Plate Kit (Beacon Analytical Systems Inc). As shown in Figure , DON production by *F. graminearum* remained low in the first two weeks after inoculation where the toxin concentration did not reach above 0.5 ppm. The concentration of DON increased dramatically at 28 dpi with a ~24.8 fold increase in concentration compared to 14 dpi. At its highest level, DON concentration reached 66.7±Xppm at the latest time point sampled, which was at 31 dpi. The increase in DON accumulation at 28 dpi is consistent with the increase in

biomass of the wild type compared to the *Tri5* mutant shown in Figure suggesting that these two processes may be linked.

Discussion

The experiments described in this chapter on crown rot disease symptom severity and fungal biomass accumulation indicated that the *Tri5* gene and DON toxin of *F. graminearum* plays a role in colonisation in the late stage of phase 2 of infection in wheat. The studies that were described in Chapter 5 indicated that the PH-1 Tri5 mutant showed no difference in symptom development compared to the wild type but showed reduced fungal biomass accumulated at the single assessment point of 28 dpi. A similar result was obtained here with an Australian isolate at the same time after inoculation. These results are consistent with a previous study that found that FCR of an American wild type isolate (Gz3639) and its *Tri5* deletion mutant (Mudge *et al*., 2006) where the *Tri5* mutant produced similar disease symptoms to wild type but unlike its wild type it could not be isolated from upper nodes. All of these studies suggest that *Tri5* mutants have reduced colonisation ability and that the trichothecene toxin must play some role in permitting fungal colonisation in the host. This has parallels with the role for DON in FHB where it is required to colonise the rachis and spread through the head (Jansen *et al*., 2005).

Considering the infection pathway of crown rot (Chapter 2) the role for DON as a colonisation factor late in phase 2 can be understood. At phase 1 (2 dpi) the fungal spores are germinating on the stem surface, at phase 2 (14 dpi) *F. graminearum* has colonised the outer leaf sheath of the wheat plant and at 28 dpi it is transitioning from phase 2 to phase 3 of infection where it moves toward the middle of the stem to rapidly colonise the plant crown tissue. Transcription of the *Tri5* gene was higher at 2 dpi compared to axcenically cultured mycelia, therefore initial contact with the plant, which includes spore adhesion, germination and penetration may initiate DON production. When the concentration of DON rises to 28.27ppm at 28dpi in the wild type, we observe an increase in fungal biomass. We see an increase in biomass of the *Tri5* mutant at this time point but not to the same degree as the wild type. It may be reasonably explained that DON aids the increase in fungal biomass at the end of phase 2 but not late in phase 3 when considering the plant tissue that *F. graminearum* is trying to colonise at the respective time points. At the end of phase 2 the fungus has reached the base of leaf sheath 1 and is attempting to colonise to the center of the stem including. At the end of phase 3 the fungus has already reached the pith of the crown tissue and is easily colonised. Therefore maybe when the fungus is invading into a new area and actively

moving through physical barriers up or down the stem such as at the end of phase 2 or as observed in (Mudge *et al*., 2006) it will encounter additional physical barriers such as epidermal layers and stem nodes and this is where DON may greatly facilitate an increase in fungal biomass.

Gene expression analysis by RT-qPCR and the DON quantification by ELISA revealed that the *Tri5* gene was expressed to similar levels throughout the infection, but large quantities of DON was not detected until 28 dpi. If one was free to speculate it seems that DON is a chemically stable toxin (Widestrand & Pettersson, 2001) therefore it may have a cumulative effect in the plant tissue. Because of the small amount of fungal biomass present during phase 1 and 2 of infection, DON must accumulate slowly to begin with and at 28 dpi enough DON has been amassed to enable the fungus to colonise through the stem base into the crown tissue where the fungus can colonise freely and dramatically increase its biomass. DON would assist fungal colonisation through its action of inhibiting plant protein synthesis and inducing programmed host cell death (Desmond *et al*., 2008b). Wheat requires relatively high concentrations of DON (50-100 ppm) to accumulate to inhibit protein synthesis and induce cell death (Desmond *et al*., 2008b). Thus concentrations of DON that impair host functions throughout the entire tissue may not accumulate until late in phase 2 and early in phase 3 of infection. Once this threshold is reached the fungus may then be able to rapidly colonise the surrounding tissue, accounting for the rapid increase in fungal biomass observed at this time. This is consistent with the different rates of fungal colonisation observed at the 28 dpi time point.

Presumably the fungus employs other mechanisms to assist colonisation as evidenced by the recovery of the *Tri5* mutant's colonisation by 31 dpi. Using the *Tri5* mutant we observed that FCR disease symptoms still develop in the absence of DON production and this is consistent with the observations of Mudge *et al* (2006) and Chapter 2. Lesion development may be the result of host reactions such as the induction of phenolic compounds that are frequently observed in defence responses. Plant defence genes e.g. chitinases, peroxidases, were induced early on during the crown rot infection experiments conducted in this chapter, usually within 2 dpi (Desmond, 2008, Desmond *et al*., 2005). Interestingly, the induction of these plant defence genes was independent of the fungus ability to produce DON as no difference were observed in between the wild type and *Tri5* mutant in host defence gene induction early in infection (Desmond *et al*., 2008b). We know from Chapter 2 that during phase 2 (14 dpi) *F. graminearum* biomass is declining and that the fungus is expressing a suite of detoxification genes possibly in response to these plant defences. The time between the first induction of the *Tri5* gene (2 dpi) and the increase in DON concentration (28 dpi)

may represent a vulnerable time in the infection cycle and may be an opportunistic time to target *F. graminearum* for biological, genetic and chemical control methods.

Experimental Procedures

FUNGAL STRAINS AND INOCULUM PREPARATION

All experiments described here were conducted with the Australian *F. graminearum* isolate CS3005 (Akinsanmi *et al.*, 2006) and the *Tri5* deletion mutant (Δ*Tri5Fg*) (Desmond *et al*., 2008b). Macroconidia for inoculation were produced in the same way as Chapter 2.

PLANT GROWTH, INOCULATION AND HARVESTING TECHNIQUE.

All seedlings were grown in a glasshouse and then transferred to a controlled facility for inoculation as in Chapter 3.

SCORING FCR DISEASE SEVERITY

Crown rot disease severity of Kennedy seedlings that were inoculated with *F. graminearum* PH-1 wild type and Δ*Tri5Fg*(PH-1) was calculated using the scoring system outlined in Chapter 3.

DNA EXTRACTIONS AND *F. GRAMINEARUM* BIOMASS ESTIMATIONS

For each time point three biological replicates were taken in parallel. Each biological replicate comprised a pool of 8-10 shoot bases. Shoot bases were ground in liquid nitrogen with a mortar and pestle and genomic DNA was extracted and fungal biomass estimated by the same method as in Chapter 2.

RNA EXTRACTION AND GENE EXPRESSION ANALYSIS BY RT-QPCR

Harvested infected wheat tissue that was used for fungal biomass estimations was also used as a source of RNA and gene expression analysis. Total RNA was extracted from this tissue using a QIAGEN RNeasy® Plant Mini Kit in the same manner as Chapter 2. Total RNA was then reverse transcribed forming cDNA using SuperScript III according to manufacturers instructions. To find how abundant the *Tri5* gene transcript was RT-qPCR was performed using the same reaction volumes and cycling conditions as shown about for biomass estimations. Primers used to amplify *Tri5* and *F. graminearum* 18S can be found in (Desmond *et al*., 2008b).

APPENDIX 4:

Supplementary Table 1. Functional classes of *F. graminearum* genes down-regulated during CR that are statistically enriched compared with the whole genome. Gene functional category was assigned using FunCatDB (MIPS) and a P-value was calculated using the hypergeometric distribution.

2 Days postinoculation	*P*-value	14 Days postinoculation	*P*-value	35 Days postinoculation	*P*-value
Metabolism	0	Metabolism	0	Biosynthesis of glycine	3.95E-02
Energy	7.32E-05	Energy	8.69E-11	nitrogen, sulfur and selenium metabolism	1.33E-02
Cell Cycle and DNA Processing	1.38E-12	Cell Cycle and DNA Processing	5.00E-11	Urea Catabolism (not urea cycle)	
Transcription	2.56E-11	Transcription	4.18E-10	Regulation of Nitroen Sulfur and Selenium Metabolism	2.05E-02
Protein Synthesis	0.022	Protein Synthesis	5.63E-20	Regulation of Nitrogen Metabolism	4.87E-02
Protein Fate	1.33E-45	Protein Fate	5.80E-51		
Protein With Binding Function or Cofactor Requirement	7.12E-24	Protein With Binding Function or Cofactor Requirement	0		
Regulation of Metabolism and Protein Function	1.94E-08	Regulation of Metabolism and Protein Function	3.64E-12		
Cellular Transport, Transport Facilities and Transport Routes	1.58E-31	Cellular Transport, Transport Facilities and Transport Routes	2.12E-44		
Cellular Communication/signal Transduction Mechanism	5.63E-10	Cellular Communication/signal Transduction Mechanism	1.60E-08		
Cell Rescue, Defense and Virulence	7.97E-13	Cell Rescue, Defense and Virulence	2.08E-19		
Interaction With The Environment	1.99E-11	Interaction With The Environment	7.54E-18		
Cell Fate	2.52E-12	Cell Fate	2.59E-15		
Development (systemic)	0.03				
Biogenesis of Cellular Components	2.07E-21	Biogenesis of Cellular Components	5.04E-24		
Cell Type Differentiation	1.00E-17	Cell Type Differentiation	1.00E-17		
Organ Differentiation	0.015				

Supplementary Table 2. *F. graminearum* Genes whose expression is significantly up-regulated in both CR and FHB

Gene ID	Alias	Description
fg00011	fg12416	Fungal transcriptional regulatory protein
fg00042	fgd2-360	non-ribosomal peptide synthetase (NPS8)
fg00449	fgd18-40	WD repeat protein IEF SSP 9502
fg00517	fg12634	conserved hypothetical protein
fg01262	fg12925	hypothetical protein
fg01290	fgd65-430	mitochondrial ribosomal protein L2 of the large subunit
fg01427	fg13154	hypothetical protein
fg01521	fg13182	conserved hypothetical protein
fg01579	fgd81-70	hypothetical protein
fg01887	fg13288	conserved hypothetical protein
fg01980	fgd108-30	conserved hypothetical protein
fg02120	fgd111-490	hypothetical protein
fg02153	fg13384	conserved hypothetical protein
fg02568	fgd128-200	hypothetical protein
fg02580	fgd128-320	maltose permease (MalP)
fg02653	fgd132-490	hypothetical protein
fg02792	fgd141-10	NAD(P)H-dependent oxidoreductase
fg03143	fgd148-690	Glycosyl hydrolase.
fg03190	fgd148-1260	hypothetical protein
fg03682	fgd161-130	conserved hypothetical protein
fg03965	fgd168-1080	conserved hypothetical protein
fg04114	fgd179-390	adenosine deaminase
fg04132	fgd179-600	RNA helicase dbp2 (dead box protein)
fg04461	fgd192-170	hypothetical protein
fg04768	fgd196-1000	endo-1,3-beta-glucanase
fg04930	fgd199-440	alpha-mannosidase 1a (glycoside hydrolase) processes glycoproteins)
fg05018	fgd202-60	hypothetical protein
fg05395	fgd218-110	conserved hypothetical protein
fg05694	fgd233-70	hypothetical protein
fg06445	fgd259-190	endo-1,4-beta-xylanase
fg07037	fgd293-110	protein
fg07213	fgd303-270	dolichol phosphate-mannose biosynthesis regulatory protein DMP2)
fg07765	fgd318-1210	isotrichodermin C-15 hydroxylase (cytochrome P-450 monooxygenase CYP65A1)
fg07832	fgd320-720	CCC1 protein (involved in calcium homeostasis) probably essential
fg08021	fgd323-710	conserved hypothetical protein
fg08030	fgd323-830	hypothetical protein
fg08126	fgd328-40	alpha glucosidase II beta subunit. Mannose-6-phosphate receptor.
fg08172	fgd328-550	Cu-binding metallothionein
fg08269	fgd329-1170	hypothetical protein
fg08613	fgd348-410	OPI3 - methylene-fatty-acyl-phospholipid synthase
fg08765	fgd354-80	hypothetical protein
fg09349	fgd383-50	hypothetical protein
fg09355	fgd383-130	hypothetical protein

fg09464	fgd387-160	conserved hypothetical protein
fg09923	fgd411-540	conserved hypothetical protein
fg09972		conserved hypothetical protein
fg10058	fgd417-460	hypothetical protein
fg10093	fgd417-880	hypothetical protein
fg11251	fgd463-30	hypothetical protein
fg12051	fgd168-1280	DHA14-like major facilitator efflux transporter (MFS transporter)
fg12087	fgd196-280	beta-glucosidase
fg12091	fgd196-370	hypothetical protein
fg12334		hypothetical protein
fg12399	fgd148-200	high affinity methionine permease
fg12678	fgd29-230	Spore coat protein SP96 precursor
fgd100-50	fg01866	conserved hypothetical protein
fgd10-70	fg00243	predicted short protein
fgd107-180	fg13319	xylanase (c-terminal fragment)
fgd108-10	fg13351	endo-1,4-beta-xylanase A precursor
fgd112-460	fg02181	predicted protein
fgd113-110	fg02202	endoglucanase IV precursor
fgd117-440	fg02386	pectate lyase
fgd117-80	fg02354	conserved hypothetical protein
fgd122-130	fg02414	predicted protein
fgd122-80	?	predicted protein
fgd129-130	fg12177	predicted protein
fgd13-240	fg00354	predicted protein
fgd132-40	fg02613	predicted protein
fgd142-370	fg12962	Glycosyl hydrolase.
fgd143-160	fg02915	predicted protein
fgd144-30	fg02939	predicted protein
fgd148-480	fg03129	predicted short protein
fgd149-30	fg03194	PGU1 - Endo-polygalacturonase
fgd150-420	fg03254	predicted protein
fgd151-120	fg03315	endopeptidase K
fgd157-280	fg03441	predicted protein
fgd158-190	fg03457	Cutinase 1 precursor [EC 3.1.1.74]
fgd159-550	fg03537	TRI5 trichodiene synthase
fgd159-600	fg03543	TRI14 trichothecene biosynthesis gene
fgd160-820	fg03632	Cellulose binding protein CEL1
fgd160-870	fg03637	thermophillic desulferizing enzyme
fgd160-980	fg03646	nicotinamide mononucleotide permease
fgd161-270	fg03695	endoglucanase IV precursor
fgd168-1110	fg03968	cellulose binding protein CEL1 (maybe endoglucanase?)
fgd190-190	fg04390	protein
fgd192-540		predicted protein
fgd192-620	fg04498	predicted protein
fgd193-250	fg04580	ABC1 transport protein g)
fgd193-690	fgs00260	predicted protein
fgd194-400	fg04647	gEgh 16 protein (hyphae formation)
fgd194-500	fg04656	conserved hypothetical protein
fgd196-160	fg12982	glucoamylase precursor. .
fgd196-690	fg12996	predicted protein
fgd198-50	fg12173	sterol glucosyltransferase
fgd199-340	fg04918	predicted protein

fgd200-540	fg04971	predicted protein
fgd201-290	fg05010	predicted protein
fgd203-80	fg05046	predicted protein
fgd212-740	fg05278	amidophosphoribosyl transferase
fgd22-210	fg00544	predicted protein
fgd225-50	fg05483	ARG8- acetylornithine aminotransferase
fgd233-70	fg05694	predicted protein
fgd238-130	fg06017	conserved hypothetical protein
fgd242-330	fg06106	predicted protein
fgd254-340	fg06331	zinc transporter
fgd257-190	fg06397	endoglucanase B
fgd259-1040	fg06506	predicted protein
fgd259-1490	fg06546	predicted protein
fgd259-420	fg06466	conserved hypothetical protein
fgd259-880	fg06493	conserved hypothetical protein
fgd266-270	fg06658	conserved hypothetical protein
fgd276-10	fg06796	centractin (ARP1) has a Ran binding doamin zn-finger domain
fgd292-290	fg07026	predicted protein
fgd30-160	fg00656	F1F0-ATP synthase subunit G
fgd302-160		predicted protein
fgd303-450	fg07227	isp4 protein
fgd313-180	fg07511	predicted protein
fgd315-140	fg07574	predicted protein
fgd318-1230	fg07767	predicted protein
fgd324-140	fg08081	gibberellin 20-oxidase
fgd328-320	fgs00473	predicted protein
fgd333-70	fg08322	predicted protein
fgd349-530	fg08683	transcriptional regulator atrx homolog
fgd366-30	fg08953	predicted protein
fgd366-50	fg08954	conserved hypothetical protein
fgd367-180		predicted protein
fgd367-740	fg09013	predicted protein
fgd374-70	fg09291	pectate lyase 1
fgd37-520	fg05944	predicted protein
fgd377-30	fg09301	predicted protein
fgd383-430	fg09382	alkaline protease (oryzin).
fgd383-90	fg09353	gEgh 16 protein (hyphae formation)
fgd384-70	fg09390	conserved hypothetical protein
fgd401-250	fg09682	predicted protein
fgd401-920	fg09733	20S proteasome maturation factor.
fgd406-570	fg09820	cysteine dioxygenase type I.
fgd417-650	fg10073	conserved hypothetical protein
fgd441-210		predicted protein
fgd450-170	fg10791	ATP-dependent RNA helicase DHH1
fgd452-230	fg10863	conserved hypothetical protein
fgd458-390	fg11064	glycine-rich RNA-binding protein
fgd458-410	fg13105	beta-mannanase
fgd458-720	fg11095	carbonic anhydrase
fgd4-60	fg00062	TOX 3 KP4 killer toxin
fgd460-780	fg11209	bromodomain protein BDF1
fgd462-80	fg11245	predicted protein
fgd464-830		conserved hypothetical protein
fgd465-390	fg11366	conserved hypothetical protein

fgd468-280	fg11488	Cellulose binding protein CEL1
fgd470-70	fg11496	predicted protein
fgd52-70	fg12837	predicted protein
fgd53-160	fg12859	UTR1 protein, associated with ferric reductase activity
fgd5-700	fg00150	endo-polygalacturonase 6
fgd64-70	fg01253	predicted protein
fgd65-690	fg01309	ethanolamin kinase
fgd66-100	fg13125	predicted protein
fgd73-80	fg13160	predicted protein
fgd75-100	fg01468	conserved hypothetical protein
fgd79-260	fg01570	Cutinase precursor [EC 3.1.1.74]
fgd91-480	fg01728	predicted protein
fgd92-100	fg13247	predicted protein
fgd94-90	fg01831	Trihydrophobin precursor
fgs00075	fg12889	predicted protein
fgs00081	fgd62-280	predicted short protein
fgs00199	fgd160-180	predicted short protein
fgs00260	fgd193-690	predicted short protein
fgs00299	fgd209-50	predicted short protein
fgs00399		predicted short protein
fgs00411		predicted short protein
fgs00599		predicted short protein
fgs00630	fgd464-230	predicted short protein
fgs00658		predicted short protein